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JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

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Enhancement of Penicillin Effectiveness by Traces of Cobalt*

By LOUIS A. STRAIT, JEAN DUFRENOY, and ROBERTSON PRATT, with the Laboratory Assistance of VIRGINIA LAMB†

Addition of trace amounts of cobalt to the nutrient agar in test plates increases approximately four- to eightfold the effectiveness of relatively dilute penicillin solutions in producing inhibition zones on plates seeded with *Staphylococcus aureus* and decreases to approximately one-third or less, depending upon the test organism that is used, the minimum or threshold concentration of penicillin that can be detected by the cylinder-plate technic.

DURING the course of studies of the cytochemical action of penicillin on bacteria (1, 2) and of the interrelationship between the biological and the physical phenomena involved in the assay of penicillin by the cylinder-plate method (3) we have been led to test the effect of trace amounts of cobalt on penicillin activity (4), and we have observed that the effectiveness of penicillin can be markedly increased by proper use of suitable concentrations of cobalt. Addition of trace amounts of cobalt to the nutrient agar in test plates increases approximately four- to eightfold the effectiveness of relatively dilute penicillin solutions in producing inhibition zones on plates seeded with *Staphylococcus aureus* and decreases to approximately one-third or less, depending upon the test organism that is used, the minimum or threshold concentration of penicillin that can be detected by the cylinder-plate technic.

EXPERIMENTAL

Results of a typical experiment in which different amounts of cobalt chloride were incorporated in the test agar are plotted in Fig. 1A. Ordinates represent zone diameters corresponding to the different concentrations of penicillin plotted on the abscissae. The data are from plates seeded with *Staphylococcus aureus* NRRL 313 (same as FDA strain 209P) and processed by the three-hour technic described earlier (3). The penicillin used throughout this work was a crystalline preparation of sodium penicillin G that assayed 1560 u./mg. The concentration of 1 mg. $CoCl_2 \cdot 6H_2O$ per L. has been most effective for inducing the enhancing action in our experiments,

* Received Oct. 16, 1947, from the University of California College of Pharmacy, The Medical Center, San Francisco, Calif.

† Acknowledgment is made to the Cutter Laboratories, Berkeley, Calif., for a generous grant that supported a portion of this research.

although considerable effect has been observed with concentrations as low as 0.1 mg./L. and as high as 10 mg./L. A concentration of 50 mg./L. was without effect on the size of the inhibition zone produced by penicillin. It should be noted that although the curves plotted in Fig. 1A are all linear, there is a slight tendency toward convergence at the higher concentrations. This seems to indicate that the enhancing action of the cobalt is greater at the lower concentrations of penicillin in the range plotted.

The enhancing effect of the cobalt was revealed also by the shorter period of incubation that was required for the development of discernible zones of inhibition around cylinders containing penicillin when a trace of cobalt was added to the test agar than when cobalt was omitted. This may be interpreted as evidence of the lower effective threshold concentration of penicillin in the presence of trace amounts of cobalt, since the rim of the inhibition zone represents the limit of diffusion of a bacteriostatic or bactericidal concentration of penicillin, and there is no reason to assume a higher rate of diffusion of penicillin on plates containing cobalt than on the standard plates. Striking evidence of the lowered threshold concentration of penicillin required for inhibition of bacteria in the presence of appropriately small concentrations of cobalt is afforded by results from plates seeded with a penicillin-resistant organism. On test plates seeded with *Escherichia coli* and incubated for sixteen hours at 37°, 10 u./ml. was the lowest concentration of penicillin that produced a detectible and readable zone on media to which no cobalt had been added. The average diameter of the zones was about 12 mm. However, when the test agar contained in addition to the standard nutrients cobalt chloride in a concentration of 0.1 mg./L., penicillin solutions containing only 1 u./ml. produced zones with an average diameter of 15 mm. in the same time, and on media containing 1 mg. cobalt chloride per liter the same solution of penicillin produced zones that averaged 16 mm. in diameter.

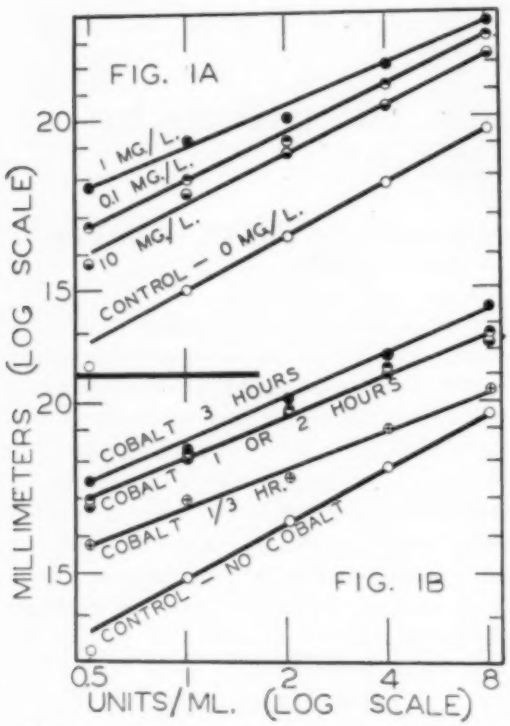

Fig. 1A.—Diameters of zones of inhibition corresponding to different concentrations of penicillin on test plates seeded with *Staphylococcus aureus* and containing different amounts of cobalt chloride in the nutrient agar. Plates were processed by the three-hour technique involving physical development.

Fig. 1B.—Diameters of zones of inhibition corresponding to different concentrations of penicillin on test plates seeded with *Staphylococcus aureus* and flooded with a solution containing 1 mg. of $CoCl_2 \cdot 6H_2O$ per L. for different lengths of time before addition of the penicillin. (See text for explanation.) Preincubation period was three hours for all cultures. Secondary incubation period was three hours for control cultures; two hours for all others.

Our evidence suggests that the action cannot be considered merely as the additive effect of two antibiotic agents acting simultaneously. No inhibition zones developed when solutions containing 0.1 or 10 mg. $CoCl_2 \cdot 6H_2O$ per liter were placed in cylinders on standard test plates, and mixtures of solutions of cobalt chloride and of penicillin (in the concentrations tested) produced no larger zones on standard test media than did solutions containing the same amount of penicillin alone.

The evidence does indicate that the effect may be due, in part at least, to a kind of "sensitization" of the bacteria, since it is clear from the following experiments that the duration of the contact of the cobalt solution with the organisms is important. In one set of experiments plates were prepared and seeded with *Staphylococcus aureus* as for the FDA cylinder-plate assay, which is essentially similar to that recommended by Schmidt and Moyer (5). Then they were preincubated (without penicillin) for three hours, during which time 2 ml. of a solution containing 1 mg. $CoCl_2 \cdot 6H_2O$ per liter were flooded over different sets of plates for varying fractions of the three-hour period. For example, one set of plates was exposed to the solution for the full three hours of preincubation. Another set was in contact with the cobalt solution during only the last two hours of the preincubation. Other sets were exposed only during the last hour or the last twenty minutes. At the end of the preincubation the residual of the cobalt solutions was carefully siphoned from the plates and penicillin solutions (0.5 to 8 u./ml.) were placed in the cylinders on each set of plates. All plates were then reincubated for two hours after which time they were processed by the physical development technic (3). The results of an experiment of this series are plotted in Fig. 1B. It is clear that a short exposure (twenty minutes) was sufficient to produce an appreciable enhancing

effect. Greater enhancement occurred asymptotically as the period of contact was extended up to three hours.

Results obtained in serial dilution experiments support the conclusions drawn from plate assays. Only one-half as much penicillin or less was required to prevent the development of turbidity in cultures of *Staphylococcus aureus* in broth containing 1 or 10 mg. of cobalt chloride per liter as in the same broth lacking cobalt. No bacteriostatic or bactericidal effect was observed in broth containing similar concentrations of cobalt but no penicillin.

In other experiments the turbidity of broth cultures containing different amounts of cobalt but no penicillin was estimated at half-hour intervals for the first six hours after inoculation. It was observed that trace amounts of cobalt added to the broth reduced the duration of the lag period of slow growth that occurs before the onset of the rapid rate of multiplication which characterizes the logarithmic phase of growth. In the absence of cobalt the onset of the log phase in cultures of *Staphylococcus aureus* required about three hours but in the presence of 0.1, 1, or 10 mg. cobalt chloride per liter the lag period before inception of rapid growth was shortened appreciably (one-half hour or more). Further evidence of stimulation of growth by subbacteriostatic concentrations of cobalt is provided by the ring of enhanced growth that develops around cylinders from which solutions of cobalt are permitted to diffuse on standard test plates (no cobalt added to the medium). The distance from the cylinder to the ring of denser growth depends, of course, on the concentration of the cobalt solution. When toxic concentrations are used, the ring of enhanced growth is seen bounding the clear zone of inhibition which represents the limit of diffusion of bacteriostatic concentrations of cobalt. In our experiments the minimal toxic concentration for *Staphylococcus aureus* was at least one hundred times the concentration that was optimal for producing the enhancing effect on penicillin activity. A similar ring of enhanced growth in the presence of subbacteriostatic concentrations of cobalt was pictured clearly by Herrero (6).

The growth stimulating effect of trace amounts of cobalt appears significant in view of the fact that it has been recognized for some time that penicillin exerts its greatest antibiotic effect on susceptible organisms when they are in the logarithmic phase of their growth curve. It has been reported that penicillin acts best on bacteria in an environment which promotes their multiplication (7) and that conditions which increase the rate of growth of bacteria increase the rate at which penicillin acts (8). It may be postulated tentatively that the "sensitization" of the bacteria by suitably low concentrations of cobalt is due, in some measure at least, to a stimulation of metabolism and/or growth that tends to place the organisms in a physiological condition in which they are more readily susceptible to the action of penicillin.

Our observations agree fundamentally with those recorded in the literature but appear to be at variance with the conclusions, recently reported, concerning the inhibitory action of cobaltous ion on bacterial metabolism (9). It should be observed, however, that the report is based on experiments in the toxic range of cobalt concentrations: the lowest concentrations employed in that work were approximately 230 times greater than the trace concentration that produced the greatest stimulation in our work.

Similar effects of cobalt in increasing the effectiveness of penicillin have been observed *in vitro* with *Staphylococcus aureus* NRRL 313, *Bacillus subtilis* NRRL B-558, *Escherichia coli*, and *Proteus vulgaris* HX19. The most pronounced effect was observed on plates seeded with *Proteus vulgaris* which seems to be especially sensitive to cobalt. The possibility of improving the effectiveness and the clinically useful antibiotic spectrum of penicillin *in vivo* by proper use of cobalt has been demonstrated in mice (10). A broader study directed at extending and improving the technic is under investigation with different subjects.

SUMMARY

1. Addition of $CoCl_2 \cdot 6H_2O$ in concentrations of 0.1 to 10 parts per million to the test agar used in assaying penicillin by the cylinder-plate technic causes pronounced increases in the diameters of the zones of inhibition that are formed on plates seeded with *S. aureus*, *E. coli*, *P. vulgaris*, and *B. subtilis*. The effectiveness of penicillin, as measured by this test, is increased two- to fourfold

2. Interpretations of the data and possible therapeutic applications are discussed briefly

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Antibacterial Activity of Combinations of Iodine and Human Blood Fractions*,†

By LOUIS GERSHENFELD and BERNARD WITLIN

Iodine was combined with whole citrated human blood and with several fractions of this material. Physical and bacteriostatic properties of these iodine compounds were determined and the results are reported.

GERSHENFELD and Miller (1) previously reported that mixtures of whole blood with iodine solutions resulted in the taking up of the free iodine by the blood and under certain conditions there was a retention of antibacterial efficiency by the blood-iodine mixture.

Iodinated horse serum has been used as a chemotherapeutic agent in various bacterial diseases. Very little information as to the exact method of preparation and to its therapeutic value is available. One marketed preparation, Iodalbin,[1] recommended for parenteral use is reported as containing 21.5 per cent iodine combined with the horse serum proteins. Muus, Coons, and Salter (2) prepared iodinated horse serum albumin and reported that the amount of iodine which entered into the protein molecule increased with the addition of iodine up to the maximum, which they reported to be 15 per cent. The exact changes produced in various proteids by iodination have not been determined but distinct differences have been observed.

This study has been undertaken in an attempt to note if the various human blood-iodine combinations possess bacteriostatic or bactericidal efficiencies and to determine if possible the blood fractions which combine to form such bacteriostatic or bactericidal compounds.

EXPERIMENTAL

The following whole human blood and fractions thereof were employed: citrated whole blood; erythrocytes washed free of citrated plasma; dried human blood cells[2]; citrated normal human plasma[3] (65 Gm. protein/L.); fibrinogen[3] (3 Gm. protein/L); gamma and beta globulins[3] (20 Gm. protein/L.); alpha globulin and lipids[3] (5 Gm. protein/L.); alpha and beta globulins[3] (4.5 Gm. protein/L.); albumin[3] (29 Gm. protein/L); and albumin and alpha globulin[3] (1 Gm. protein/L.).

Test for Bactericidal Efficiency of Iodine-Blood Protein Mixtures.—To 10-cc. volumes of each of the above solutions, suspensions or powders, a 5% aqueous iodine-potassium iodide solution (Lugol's) was added in varying amounts at 22.5°.

Bactericidal efficiency tests were performed in accordance with the Food and Drug Administration technic (3) employing *Staphylococcus aureus* at 37°.

The iodine-blood and iodine-blood fraction mixtures were tested for bactericidal efficiency immediately after preparation and also on the first, second, third, fourth, and fifth day thereafter.

Findings.—In all instances, iodine-treated citrated human blood, hemoglobin, plasma and its fractions failed to reveal bactericidal efficiencies other than those which could be attributed to the presence of free iodine.

Absorption of Iodine by Blood and Its Derivatives at Varying Temperatures and the Testing of the Resulting Products.—To 250 cc. portions of human citrated blood and of the various fractions already described, there were added 250 cc. distilled water and 18 cc. of 50% aqueous iodine solution (10 X Lugol's). One series of each was prepared at each of the following temperatures: 22.5°, 37° and 56°.

The precipitates formed were washed with distilled water until negative for free iodine. They were dried at room temperature and tested for bacteriostatic efficiency by the F.D.A. Agar-Plate technic employing *S. aureus* at 37°. The results are reported in Table I.

Findings.—(*a*) Larger amounts of iodine went into combination with blood and its derivatives at 56° than at 37° and more at 37° than at 22.5°.

(*b*) Washed precipitates of blood-iodine mixtures prepared at 56° possessed greater bacteriostatic properties (as revealed by inhibition zones) than those prepared at 37° or 22.5°.

(*c*) Precipitates prepared by the combination of iodine with albumin fractions, plasma or with egg albumin and washed until all the free iodine had been removed did not display bacteriostatic proper-

* Received Sept. 26, 1947, from the Philadelphia College of Pharmacy and Science, Philadelphia, Pa.
Presented to the Scientific Section, A. PH. A., Milwaukee meeting, August, 1947.
† This investigation was conducted at the Philadelphia College of Pharmacy and Science, under a grant from the Iodine Educational Bureau's Fellowship at Mellon Institute.

[1] "Iodalbin," Parke, Davis & Company.

[2] "Lyocyte" Powder, Sharp & Dohme, Inc.

[3] Supplied through the courtesy of the Department of Physical Chemistry, Harvard Medical School, and prepared under contract between the Office of Scientific Research and Development and Harvard University.

ties (as revealed by the nonproduction of inhibition zones).

(*d*) Larger inhibition zones were obtained with iodine-alpha and beta globulin mixtures than with alpha globulin and lipids or gamma and beta globulins or any of the other fractions.

Bacteriostatic Efficiency of Iodine-Plasma Compounds Prepared by the Gradual Addition of 10 X Strength Lugol's Iodine Solution to Aqueous Dilutions of Human Blood Plasma and Its Fractions (see Table II).—(*a*) To 250 cc. citrated human blood plasma there were added 250 cc. distilled water in a 1000-cc. beaker. The latter, containing the diluted plasma, was kept in a water bath at the desired temperature and constantly stirred with an electrically operated stirring apparatus. Ten X strength Lugol's iodine solution was added in 0.1-cc. amounts until free iodine was no longer present. The mixture was filtered and the precipitate was washed with distilled water until negative for free iodine (see Table II). Precipitates were then examined for bacteriostatic efficiency by the F.D.A. Agar-Plate technic against *S. aureus* at 37°.

TABLE I.—BACTERIOSTATIC EFFICIENCY OF IODINATED HUMAN BLOOD, PLASMA AND PLASMA FRACTIONS AT VARYING TEMPERATURES

Substance	Mixture made at Temp. ° C.	Washed Precipitate (Iodine-Free) F.D.A. Agar-Plate (*S. aureus*) Zone in Mm.
Whole blood (citrated)	22.5	12
	37.0	15
	56.0	17
Hemoglobin	22.5	12
	37.0	15
	56.0	17
Plasma (citrated)	22.5	5
	37.0	8
	56.0	10
Fibrinogen	22.5	5
	37.0	5
	56.0	8
Gamma and beta globulins	22.5	5
	37.0	6
	56.0	8
Alpha and beta globulins	22.5	15
	37.0	17
	56.0	20
Albumin	22.5	0
	37.0	0
	56.0	0
Albumin and alpha globulin	22.5	0
	37.0	0
	56.0	2
Alpha globulin and lipids	22.5	5
	37.0	7
	56.0	8
Fibrin foam[a]	22.5	0
	37.0	0
	56.0	0
Controls		
Iodalbin	..	10
Egg albumin	22.5	0
Egg albumin	37	0
Egg albumin	56	0
2% aqueous iodine	...	10 (cup plate)
5% aqueous iodine	...	10 (cup plate)
2% iodine tincture (U. S. P.)	...	16 (cup plate)
7% strong iodine tincture (N. F.)	...	16 (cup plate)

[a] Fibrin foam, Sharp & Dohme, Inc.

TABLE II.—AGAR-PLATE TEST. INHIBITION ZONES OBTAINED WITH IODINE-HUMAN BLOOD PLASMA COMPOUNDS PREPARED AT VARYING TEMPERATURES

Compound Prepared Using 250 Cc. Plasma and 250 Cc. Distilled Water with 10 X Lugol's Solution at Temp., ° C.	10 X Lugol's Used, Cc.	Zone of Inhibition (*S. aureus*), Mm.
22.5	1.5	1.0
37	6.0	2.5
45	12.0	5.0
56	21.5	10.0

Findings.—The amount of iodine contained in the compounds was dependent upon the temperature at which combination took place. The iodine content was greater in the product prepared at 56° than at 45°, greater at 45° than at 37°, and greater at 37° than at 22.5°.

These iodine-plasma combinations produced brown loose powders, insoluble in water, alcohol, or acid, but soluble in fixed alkalies, ammonia, and alkali carbonates. They were precipitated from their solutions by acids but went into solution with an excess of acid.

Iodine-plasma mixtures formed by the interaction between iodine and human blood plasma gave inhibition zones on F.D.A. Agar-Plate tests, the diameter of which varied depending upon the temperature which prevailed at the time of combination (or preparation).

Little is known as to the exact changes produced in proteids by iodination, but distinct differences are apparent, depending upon the temperature used and the time permitted for the iodination. The heating of a proteid solution produced a marked change upon the proteins present. Each increase in temperature affected a different proteid or group of proteids until a maximum coagulation or precipitation of proteids occurred, each responding differently to iodination.

Bacteriostatic Efficiency of Precipitates Formed by Iodine-Plasma Mixture at 22.5° and Washed Until All the Free Iodine Was Removed.—Individual precipitates were washed separately with either distilled water, 95% alcohol, benzin, carbon tetrachloride, iso-amyl alcohol, ethyl dichloride, or acetone. Individual samples of the precipitates were tested by the F.D.A. Agar-Plate technic in the wet and dry state. Samples of each of the precipitates were dried at 22.5° and 37.5°. A portion of each precipitate was then moistened with some of the solvent originally used in its preparation. The

TABLE III.—INHIBITION ZONES OBTAINED WITH IODINE-HUMAN BLOOD PLASMA PREPARED AT 22.5° AND WASHED WITH DIFFERENT SOLVENTS (*S. aureus*)

Lugol's Solution, Cc.	Plasma, Cc.	Solvent	Wet Zone, Mm.	Dried 22.5° Zone, Mm.	Dried 37° Zone, Mm.	Re-moistened Zone, Mm.
2.0	10	Distilled water	4	2	2	2
2.0	10	95% alcohol	5	0	0	0
2.0	10	Benzin	3	0	0	0
2.0	10	Carbon tetrachloride	1	0	0	0
2.0	10	Ethyl dichloride	2	0	0	0
2.0	10	Acetone	10	0	0	0
Controls		Distilled water	0	Cup plate	..	..
Controls		95% alcohol	0	Cup plate	..	..
Controls		Benzin	0	Cup plate	..	..
Controls		Carbon tetrachloride	0	Cup plate	..	..
Controls		Iso-amyl alcohol	0	Cup plate	..	..
Controls		Ethyl dichloride	0	Cup plate	..	..
Controls		Acetone	0	Cup plate	..	..

re-moistened precipitates were tested by the F.D.A. Agar-Plate technic with *S. aureus* at 37°.

Findings.—Iodine plasma precipitates prepared in distilled water at 22.5° and washed with distilled water until all the free iodine was removed displayed bacteriostatic activity when tested by the F.D.A. Agar-Plate technic. Precipitates were washed once with distilled water or the organic solvents and permitted to dry. They were then re-washed repeatedly with distilled water or the solvents until free iodine was removed. After drying, the precipitates were found to retain quantities of iodine comparable to those prepared at identical temperatures (Table II).

Iodine plasma precipitates prepared at 22.5°, washed with organic solvents, dried at 22.5° and 37° and subsequently employed in the above test did not display bacteriostatic efficiency. When the water-washed precipitates were dried at 22.5° or 37°, re-moistened with distilled water, and tested by the F.D.A. Agar-Plate technic, varying degrees of bacteriostasis were displayed as indicated by the zones of inhibition (see Table III).

SUMMARY AND CONCLUSIONS

1. Iodine combined with whole citrated human blood, hemoglobin, citrated plasma, fibrinogen, gamma and beta globulins, alpha globulin and lipids, alpha and beta globulins, and albumin and alpha globulin to form apparently stable water insoluble complex protein compounds. The iodine in combination in these compounds was readily removed by organic solvents.

2. Iodine combined with the albumin fraction of plasma and with egg albumin to form precipitates. These precipitates did not exhibit bacteriostatic or bactericidal activity after all free iodine had been removed by washing with distilled water or organic solvents

3. Larger amounts of iodine went into combination with blood and its derivatives at 56° than at 37° and more at 37° than at 22.5°.

4. Precipitates of whole citrated blood-iodine and plasma-iodine mixtures prepared at 56° and washed free of free iodine with distilled water possessed greater bacteriostatic properties than those prepared at 37° or 22.5°.

5. Precipitates of human blood plasma-iodine mixtures, filtered and permitted to dry, did not relinquish the bound iodine when repeatedly washed again with distilled water or organic solvents. Precipitates of human blood plasma-iodine mixtures repeatedly washed, immediately after their preparation, with distilled water or organic solvents readily liberated the iodine from the precipitate into the solute.

6. Greater bacteriostatic properties were obtained with iodine-alpha and beta globulin mixtures than with iodine-gamma and beta globulin mixtures or iodine-alpha globulin and lipid mixtures.

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A Quantitative and Qualitative Evaluation of Official and Unofficial Species of Psyllium Seeds and Their Mucilages*

By DAVID GREENBERG†,‡,§

A convenient method for the quantitative determination of mucilage in Psyllium seed is described. Two native unofficial Plantago seeds are described and compared with those now official.

THE following study of psyllium seeds and their respective mucilages was initiated when a sample of *Plantago wrightiana* Decne was presented to the author for comparison with the commercial psyllium seeds. The large size of the seed, which yielded a copious amount of bland-tasting mucilage, and the hardiness and high seed yield of the wild plant indicated a possible native source of psyllium seeds. Subsequent swelling tests indicated a very high percentage of mucilage. The war-born shortages of imported psyllium seed and the present national emphasis placed on providing, when possible, an economically feasible native supply of crude drugs led to a more critical investigation of *P. wrightiana* seeds in comparison with the imported psyllium seeds.

The present study of the various psyllium seeds and their mucilages was undertaken: (*a*) to provide evidence that the seeds of *P. wrightiana* are a possible source of commercial plantago seeds; (*b*) to establish a convenient and practicable means of determining the percentage content of available mucilage[1] in psyllium seeds; (*c*) to suggest a method whereby the available mucilage of psyllium seeds may be obtained in a pure form for commercial or experimental use; (*d*) to question the long-standing view that the swelling factor of psyllium seeds is indicative of the mucilage content, and to suggest that it is instead dependent on the physical properties of the mucilage; (*e*) to present some of the physical properties of mucilage solutions as a means of evaluating these products; (*f*) to determine if, within the same species of seed, the quantity of mucilage, the swelling factor, and the viscosity of definite dilutions of the pure mucilage differ significantly.

The evaluation of psyllium seeds is at present based on the National Formulary VIII *Test for Quality* in the monograph "Plantago Seed" (1). Important and more exacting test standards, as modifications of the official method, have been devised by Youngken (2). These improvements take into consideration the importance of temperature, diameter of the graduated cylinder, and a means of agitating the seeds during the test period. It will be noted, however, that these factors considered by Youngken relate to a fundamentally important property of the seed mucilage which in the final analysis determines the swelling factor. This property, the viscosity of the hydrated mucilage formed, when improperly interpreted, is undoubtedly the underlying cause for many of the discrepancies arising during swelling factor determinations made by different investigators. Clevenger (3), in one of the first attempts to evaluate psyllium seed qualitatively, used a 10-cc. cylinder and 1 Gm. of seed. The swelling factors obtained were low because of the impaction of the seed-mucilage mass within the small tube. Burlage (4), in a report on a collaborative study of the swelling factors of psyllium seeds based on the improved Youngken method, presents data which for the most part are consistent, but nevertheless display some wide discrepancies. If, as stated by Burlage, "The swelling factor is indicative of the mucilage content..." (4), such wide variation of swelling factors as 4.5 cc. within the same species is in need of explanation and, if possible, correction.

Aside from the excellence and importance of the contributions to the chemistry of the mucilages, it should be noted that the low percentages of mucilages obtained for chemical analysis by Nelson and Percival (5) and Mullen and Percival (6) leave considerable doubt as to the applicability of the information so obtained to the chemistry of the total mucilage. The 5% of mucilage obtained from the seeds studied in each case obviously represents only a portion of the available mucilage from these seeds.

* Received Sept. 30, 1947, from the College of Pharmacy, The University of Texas, Austin, Tex.
Presented to the Scientific Section, A. PH. A., Milwaukee meeting, August, 1947.
† Recipient of Kilmer Prize for 1947.
‡ Now instructor in pharmacognosy, University of Texas.
§ Acknowledgment is made to Dr. C. C. Albers, Professor of Pharmacognosy, University of Texas, for his assistance.

[1] The term "available mucilage" is used here to denote that mucilage which takes a direct part in the swelling factor and which can be obtained from the seed free of underlying seed parts.

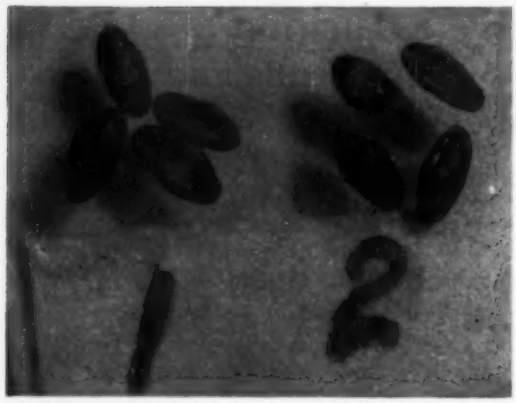

Fig. 1.—*Plantago arenaria* No. 1 and No. 2. This photograph is intended to show that these two samples are almost indistinguishable as to color, size, and shape.

Fig. 2.—*Plantago rhodosperma*. The seeds of *P. rhodosperma* are broadly ellipsoidal, pear-shaped, 2.5–3 mm. long and 1.5 mm. wide, light to dark red in color. The dorsal surface exhibits a large lighter-colored embryo broad at the small end of the seed and tapering off to a point at the edge of the broad end of the seed. The seed is bordered by a thin, translucent band. The ventral surface is flat to slightly convex. The edge is bordered by a clear band blending into the red center in which lies the conspicuous, white hilum. The two surfaces are dull and slightly reticulated.

The author assumes that the chemical investigation of seed mucilages should be made on the total mucilage from that seed or that cognizance be taken of the fact that the information obtained from the study of fractions thereof may not represent the true chemical picture. This assumption is given factual support when consideration is taken of the observations of Anderson and Fireman (7) who detected significant variations in the chemical composition of mucilage obtained from the same lot of seed with slight variations in the manner of preparing the pure mucilage. Variations in the amount of water and pressure as well as the time factor used to extract the mucilage yielded differences in the chemical constitution of the final product.

While this author is in complete agreement with Anderson, Gillette, and Seeley (8) in their recognition of the fact that a knowledge of the composition and structure of the mucilages is important as a stepping stone in the study of the more complex cell wall constituents, he again suggests that it is of importance also that the mucilage studied represent as nearly as possible the total mucilage layer, free of underlying seed parts.

MATERIALS AND APPARATUS[2]

The seeds designated in this study as *Plantago ovata* Forskal No. 1, *P. arenaria* Waldstein et Kitaibel No. 1 and No. 2 (Fig. 1) were obtained from a commercial dealer in crude drugs. *P. ovata* No. 2, *P. arenaria* No. 3, and *P. arenaria* No. 4[3] were obtained from a second commercial source. The seeds of *P. rhodosperma* Decne, identified by Dr. B. C. Tharp, Curator, Herbarium, The University of Texas (Fig. 2) were collected by hand in late June,

Fig. 3.—Herbarium specimen of mature plant of *Plantago rhodosperma*.

[2] Appreciation is also expressed to Mr. C. L. York, graduate student in Botany, The University of Texas, for his assistance in preparing all photographs.

[3] This box of seed was plainly labeled *Plantago psyllium seed*, but because of the size and shape of the seed, their swelling factors and mucilage content, and the color of the hydrated mucilage, these seeds will be designated as *P. arenaria* No. 4 in this study.

Fig. 4.—*Plantago wrightiana*. The seeds of *P. wrightiana* are elongated-ellipsoidal, boat-shaped, from 3–4 mm. in length and 1.5–2 mm. wide, pale brown with a dull reticulated surface, slightly darker brown along the margin, ending in a transparent edge. The convex dorsal surface exhibits a distinct transverse groove at the widest portion of the seed which bisects the lighter colored embryo area. The concave ventral portion is bordered by a 0.5-mm. ridge sloping into a deep concavity in the center of which lies the dark hilum surrounded by a conspicuous white border.

1946, in Austin, Tex., from plants which had been mowed down and left on the edge of a former hay field. These seeds as a whole probably do not represent the best obtainable from this plant. Figure 3 is a photograph of a herbarium sheet of *P. rhodosperma*. This plant is distributed throughout the sandy soil of Missouri and Oklahoma to Louisiana, Texas, and Arizona.

The *P. wrightiana* seeds (Fig. 4), also identified by Dr. B. C. Tharp, were obtained from plants grown in the Medicinal Plant Garden of The College of Pharmacy, The University of Texas, during 1945 and 1946. *P. wrightiana*, under cultivation, grows well on marginal and submarginal soil, favoring a deep sand, requiring little attention and no irrigation. The plant is native to Texas and Arizona and is found in dry sandy soil. When planted in October or November the mature seeds may be harvested in May or June. The fruiting spikes extend erect over the tops of the leaves with sufficient clearance for easy harvesting with a modified combine. Figure 5 is a photograph of a mature plant of *P. wrightiana* mounted in The University of Texas Herbarium.

Attempts were made to obtain samples of *P. psyllium* L., in order to complete this study on the official psyllium seeds, but none was available from the sources contacted.

Work is contemplated on the following plantago seeds native to Texas when material becomes available: *P. occidentalis* Decne, *P. helleri* Small, *P. spinulosa* Decne, *P. aristata* Michx., *P. virginica* L., and *P. inflexa* Morris.

The hand press (Fig. 6) used in the quantitative determinations reported in this study was designed by the author and tooled in the University Machine Shop. The interchangeable sieve plate allows for

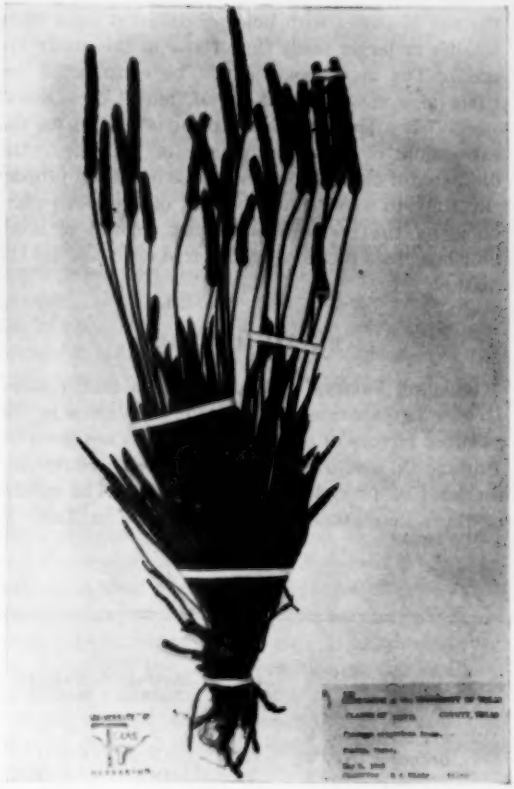

Fig. 5.—Herbarium specimen of mature plant of *Plantago wrightiana*.

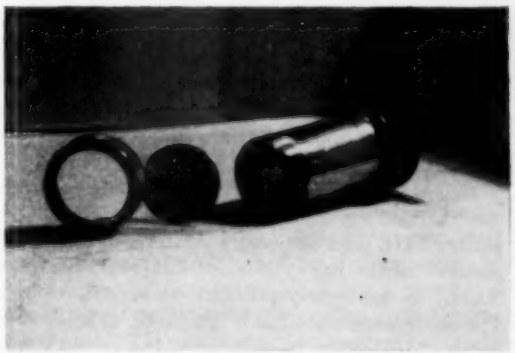

Fig. 6.—Hand Press. The hand press, except for the sieve plate, is made of stainless steel. The sieve plate is brass. The essential dimensions are as follows: The cylinder is $3^{7}/_{8}$-inches long by $1^{5}/_{8}$-inches inside diameter; thread on the $^{1}/_{2}$-inch push rod connecting the 5-inch handle to the piston are $^{1}/_{2}$-inch N.C. thread. The sieve plate is $^{1}/_{8}$-inch by $1^{53}/_{64}$-inches with approximately 200 No. 57 drill holes. The push rod is connected to the piston by a ball and sprocket joint. The piston is covered with a leather gasket press-fitted to the cylinder and screwed to the piston. The bottom ring is flanged to hold the sieve plate in place as the ring is screwed onto the cylinder.

the use of plates with holes of different sizes when smaller or larger seeds than those in this study are used. The hand press should be constructed entirely of a rust-resistant metal, monel or stainless steels. If a large size hand press is desired for the expression of larger amounts of mucilage, the diameter of the cylinder should be increased proportionately to avoid the occlusion of the sieve plate holes by the pressure of a long column of seed-mucilage mass on the mucilage-free seeds nearest the plate.

EXPERIMENTAL

Swelling Factors.—In attempts to find a satisfactory explanation for the wide variations in the swelling factors of a given lot of seeds obtained by Burlage (4) and others, the following three distinct methods of procedure were followed. The results of these determinations are presented in Table I.

TABLE I[a]

Seed	Minimum N. F. Swelling Factor, Cc.	Average Swelling Factors, Cc. Method 1	Method 2	Method 3	Percentage of Mucilage 20 Cc. H_2O	30 Cc. H_2O	Density	Absolute Viscosity in Centistokes
P. arenaria No. 1	8.0	7.0	7.0	7.0	9.00	8.16	1.0024	5.87
P. arenaria No. 2	8.0	9.5	8.0	17.0	7.66	7.36	0.9999	96.00
P. arenaria No. 3	8.0	18.0	7.0	10.0	6.95	....	1.0023	4.32
P. arenaria No. 4	8.0	18.0	6.0	14.0	8.93	....	1.0026	2.68
P. ovata No. 1	10.0	11.0	9.5	11.0	22.30	21.70	1.0023	19.38
P. ovata No. 2	10.0	13.0	10.0	13.0	20.50	....	1.0024	12.00
P. wrightiana	...	18.0	14.5	20.0	20.50	23.00	0.9934	518.40
P. rhodosperma	...	19.0	...	...	16.00[b]	15.11[b]	0.9970	Too thick

[a] All figures in this table represent averages of at least duplicate determinations.
[b] Further soaking and expression of mucilage yielded 18–20%. The high viscosity of this mucilage does not permit of complete expression under these conditions.

From these figures it is evident that the possible swelling factors obtainable from a given lot of seeds run from one extreme to another, with none having much meaning unless the method used is rigidly standardized.

Method No. 1.—Tests on 1-Gm. samples of seed were carried out as directed by the National Formulary VIII, page 336, with the interpretation of the phrase, "Shake the cylinder at intervals during 24 hours," as apparently previous workers have done. The seeds were placed in a dry 25-cc. cylinder and water added to the 20-cc. level. The cylinder was immediately inverted several times to disperse the seeds and allowed to stand for *several hours* after which time the cylinder was similarly shaken at several-hour intervals for twenty-four hours.

Method No. 2.—One gram of seed was placed in a dry 25-cc. cylinder and water added to the 20-cc. level. The cylinder is inverted *once* to disperse the seeds and the cylinder allowed to remain undisturbed for twenty-four hours, after which the reading is taken.

Method No. 3.—One gram of seed was placed in a dry 25-cc. cylinder and water added to the 20-cc. level. The cylinder is then shaken constantly and vigorously by complete inversion of the cylinder for the first hour and allowed to settle for the remaining twenty-three hours, after which the reading is taken.

It is understandable that the wholesale dealer in *P. arenaria* No. 4 (labeled as *P. psyllium*), by disregarding the macroscopic features of the seed, and without more exacting test standards, might easily interpret the high swelling factor as meeting the National Formulary minimum of 14 cc. for *P. psyllium*.

Similar differences in the results of early quantitative determinations on the same lot of seed were obtained by the author until the factor of adequate and correctly timed agitation was considered.

Quantitative Determination of Mucilage Content.—The current usage of psyllium seed mucilages, in their purified state, in medicine and industry requires a simple means of determining the quantity of mucilage available from a given lot of seed. This information is of value to the importer of the seed, and to the manufacturer of the mucilage product. The data obtainable from the pure mucilage, if they represent the total mucilage, are of value to the chemist, the doctor who prescribes the product as a laxative, and the manufacturer of cosmetics and other mucilage-containing preparations.

The proper use of the above-described hand press, which can be easily and inexpensively made in any well-equipped machine shop, will accomplish these results consistently.

The author has found that the proportion of 1 Gm. of seed per 20 cc. of water soaked for twenty hours with considerable agitation during the first hour and occasional shaking thereafter produces results which are very constant for each lot of seed and as a whole is suitable as a standard for routine determinations. The use of 30 cc. of water for each 1 Gm. of seed gave results (see Table I) which were lower in some instances and higher in others than when 20 cc. was used.

The following method was used in the determinations reported in Table I: 1.5 Gm. of seed was

macerated with 30 cc. of distilled water for twenty hours with vigorous agitation during the first hour to insure the dispersion of the mucilage formed and to break up the agglutinated clumps of seed which formed in most cases immediately upon the addition of water to the seeds. The material was then poured into the hand press, the sieve plate put in place, and the mucilage expelled by turning the handle rapidly. The thread ratio on the push rod is such as to allow rapid turning and maximum pressure with the least exertion. The adhering mucilage is removed from the outside of the sieve plate with a spatula and added to the bulk collected on a glass plate or in a tared Petri dish bottom. The mucilage is then dried in an oven or on a temperature-controlled hot plate not above 100°. The author used a steel plate 3 feet by 4 feet by $^1/_8$ inch on the underside of which was soldered 50 feet of copper tubing carrying steam. This allowed rapid drying at 95–100°. When dry the mucilage may be shaved from the glass with a single-edge razor blade and weighed, or if in a tared dish the weight may be determined directly.

As may be seen from the above description of method and the data presented in Table I, the quantitative determination of mucilage content can be easily and consistently determined. The percentages averaged and presented in Table I for each lot of seed varied for the most part within 1%.

It should be noted here that in expressing the mucilage from the seeds as described above, the seeds are left free of any mucilaginous feeling and are not crushed. A few seeds are occasionally forced through the sieve plate holes but may be easily removed when the mucilage is dry.

The question as to the completeness with which the mucilage is removed by this process was solved by re-soaking the seeds in fresh water, after the single expression, and again expelling the water. When dried this last volume of wash water yielded only a negligible amount of mucilage which can be accounted for as that small amount of mucilage remaining compressed between the seeds after the first expression. This further soaking also softens the seed and permits crushing.

Density of Mucilage Dilutions.—The determination of the density of the mucilage solutions used in determining the viscosity was necessary for computing the absolute viscosity. The data obtained are presented here because of their interesting variations. Further work is contemplated on this feature. For these determinations a dilution of 150 mg. of mucilage per 20 cc. of water was made and carried out at 28° using a 25-cc. pycnometer. A comparative study of the osmotic pressure values of the various mucilages is also contemplated.

Viscosity.—It is the viscosity values presented in Table I which show the great differences between mucilages obtained from different species of Plantago seeds as well as the vast difference between mucilages obtained from two lots of seed of the same species. These determinations were made with a Ubbelohde viscosimeter at 37.8° with dilutions of mucilage containing 150 mg. per 20 cc. of distilled water. The viscosimeter used was calibrated for water at 37.8° and the values obtained on the mucilages were correspondingly corrected.

The dried mucilage was added to the water in a test tube and heated to 100° for one to five minutes to facilitate the hydration of the mucilage, and then cooled to 37.8°. The mucilage was introduced into the viscosimeter which rested in a water bath. Determinations were made in rapid succession and an average taken. After four or five determinations, the mucilage drawn into the upper bulbs of the viscosimeter became successively less viscous, with the exception of that of the four specimens of *P. arenaria*. This is readily explained in the case of the mucilage of *P. ovata* which separates fairly rapidly, leaving a watery supernatant liquid occupying as much as one-half the volume in the test tube in a period of several days when undisturbed. The tubes of mucilage of *P. wrightiana*, *P. rhodosperma*, and *P. arenaria* No. 1 and No. 2 showed no signs of separation after one week, while those of *P. arenaria* No. 3 and No. 4 and *P. ovata* No. 1 and No. 2 separated within twenty-four hours. It is possible that the drawing of the suspension through the capillary tube causes a separation more rapidly than when standing undisturbed. Caution should be observed to avoid the introduction of bubbles into the viscous suspensions as these bubbles reduce the viscosity far out of proportion to the volume they occupy. Bubbles may be removed from the more viscous mucilage by gentle warming.

The viscosity values of seed mucilages should prove of considerable importance in evaluating the mucilage or the seed. To the manufacturer of liquid cosmetic preparations or of dry preparations intended to be wetted in their use, this factor is of utmost importance.

Description of Dry Mucilage.—The dried pure mucilage from each of the lots of seeds used in this study varied in texture and color. The mucilages of the four lots of *P. arenaria* each differ significantly from the others in color. Upon scraping the dried mucilage from the glass plate, that of *P. arenaria* No. 1 shatters into a very fine spray of glistening particles which are carried off by slight currents of air, while that of *P. arenaria* No. 2, No. 3, and No. 4 fragment without shattering or tending to be thrown into the air. The mucilages of *P. arenaria* No. 1 and No. 2 differ only slightly in color, both being a dark reddish brown with some luster. *P. arenaria* No.3 mucilage is a faint gray brown with considerable luster. That of *P. arenaria* No. 4 is a medium brown with some luster. *P. ovata* No. 1 and No. 2 mucilages are similar, both being easily removed from the glass plate in short curled strips. These strips are transparent but en masse appear to be a dull white. The mucilages of *P. rhodosperma* and *P. wrightiana* are similar to that of *P. ovata* but tend to break up into shorter strips. In all the above species the thin layer of mucilage on the plate is clear and transpar-

ent with *P. wrightiana* exhibiting denser splotches spread throughout the clear matrix. The splotches are observed to be what is apparently incompletely hydrated mucilage forcibly expressed from the seed and retaining, in a swollen, jelly-like mass, the concavity of the mucilage layer around the dorsal side of the seed.

Description of the Mucilage Dilutions.—A visual inspection of the tubes containing the dilutions of mucilage used in the viscosity and density determinations disclosed many differences. In all cases the mucilage formed is a clear, transparent liquid. The dilutions of *P. arenaria* No. 1 and No. 2 differ slightly in color, both being a light brown with a suggestion of red. *P. arenaria* No. 3 is a much lighter, dirty brown color. *P. arenaria* No. 4 is medium brown in color. The mucilages of *P. arenaria* No. 1, No. 3, and No. 4 are thin liquids allowing bubbles of air introduced when shaken vigorously to rise rapidly to the top. That of *P. arenaria* No. 2, *P. rhodosperma*, and *P. wrightiana* exhibit a ropy consistency when the tube is rotated on a horizontal plane and will retain large bubbles of air over a period of several days. The mucilage dilutions of the two samples of *P. ovata* are free flowing and do not retain bubbles of air introduced into them by shaking. The 150 mg. per 20-cc. dilution of *P. rhodosperma* yields a semisolid, nonflowing mass, very closely approaching the consistency of "set" gelatin. This was too viscous to use in the Ubbelohde viscosimeter.

DISCUSSION

The author has observed that the factors involved in the National Formulary *Test for Quality* do not comprise a simple relationship. It can readily be seen that the variables involved in the swelling factor determination, mainly the amount of agitation of the seeds in water, the time involved and frequency of agitation, the viscosity of the hydrated mucilage, the tenacity with which the mucilage adheres to the seeds as it is hydrated, the degree to which the mucilage is dispersed in the available water, and the degree to which the mucilage remains dispersed or separates, are all of considerable importance.

If the agitation of the seeds is reduced to a minimum in making the swelling factor determination, the result is an impaction of the seed-mucilage mass at the bottom of the cylinder with a considerable layer of supernatant water overlying the seeds. If the mass is agitated so as to give each seed and its mucilage content space and water in which to swell, the seeds are supported by larger areas of hydrated mucilage as well as by the viscosity of the dispersed mucilage. Again, if the mass is constantly and vigorously agitated in such a manner as to separate completely the mucilage from the seed and effect a homogeneous dispersion of the mucilage in the water, the viscosity of the mucilage and its rate of separation from the suspension will determine the depth to which the seeds sink in the cylinder. All the possible stages and their resultant swelling factor readings have been obtained by the author with the same lot of seed allowing for readings of swelling factors which are so widely divergent as to make them meaningless as regards the *Test for Quality* in the National Formulary. The widely divergent readings averaged in the collaborative reports on swelling factors presented by Burlage (4) suggest clearly the necessity of standardizing or completely revising the provisions of the swelling factor determination process.

The reproducible constants obtained by this author pertaining to percentage of mucilage content, swelling factors, and viscosities, show no direct relationship between the many possible swelling factors obtainable for a given lot of seed and the per cent of mucilage yielded by the seeds. Neither is the per cent of mucilage obtainable from a given lot of seed an indication of the viscosity of that mucilage. The wide variations exhibited by different lots of the same species of seed with regard to these important features make it possible so to misinterpret the values as to allow a poor grade of Plantago seed to be placed on the market as *P. psyllum*, e.g., *P. arenaria* No. 4, on the basis of a misleading swelling factor which meets the N. F. minimum requirements for that species, when by more exacting tests the seeds prove to have many inferior qualities.

It is not intended that the complete answer be given in this report as there are yet many factors involved concerning which the author has found no satisfactory answers. At the present writing the factor of the completeness of the hydration of the mucilage and the separation or layering of the mucilage dispersions, regardless of the viscosity, obtained from one sample of seed and no separation of the mucilage obtained from another seed has not been investigated. It should be pointed out that the accumulation of carbon dioxide resulting from the fermentation of the mucilage and the respiration of the seed which becomes entrapped in the mucilage when soaked in water over the test period is, unless controlled, sufficient to defeat the test entirely. It is of considerable interest in this connection that the seeds of *P. wrightiana* resisted fermentation for several weeks, at which time yeast colonies began to develop, and even resisted direct transplants of mycelial mats of *Aspergillus niger*. This would seem to indicate that there is present in *P. wrightiana* a substance which prevents or retards the growth of bacteria and fungi, whereas the other seeds fermented and became covered with molds within twenty-four to forty-eight hours after adding water.

SUMMARY

The present-day use of the mucilage separated from the seed of Plantago and other seeds requires a means of determining quantitatively the mucilage available from a given lot of seeds. A convenient means for this

determination is presented. From the data in Table I it is evident that the swelling factor is not indicative of the mucilage content, but is dependent on the viscosity of the mucilage formed and other related factors. It has been shown that the viscosity of the mucilage in water varies considerably in different lots of the same species of Plantago seed. It is suggested that more exacting test standards than now required be considered for the evaluation of psyllium seed. Two native Plantago seeds have been described and assayed. *Plantago wrightiana* yields a seed which in all respects compares favorably with the official seeds.

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Chemical Studies in Oklahoma Plants. VI. *Physalis mollis* Nuttall—A Plant Insecticide*,†

By LOYD E. HARRIS

Physalis mollis, **commonly known as smooth ground cherry, has been used as a fly poison. A chemical investigation of this plant has been made and its effects upon flies have been studied. The plant is effective as a fly poison and the active constituent is probably an alkaloid which has been detected.**

THE PLANT *Physalis mollis* belongs to the *Solanaceae* family. It is also known as smooth ground cherry and was described by Nuttall (1) in 1834. It grows widely throughout Oklahoma. During the early days of the state, and before the advent of prepared fly sprays, the fresh plant was used for the control of house flies. The leaves and stems were bruised, mixed with a little water and some sugar added. This was set around at convenient places and it was very effective in killing flies. A search of the literature revealed no information on the chemistry of this plant so this investigation was undertaken.

* Received Sept. 26, 1947, from The Ohio State University College of Pharmacy, Columbus.
Presented to the Scientific Section, A. PH. A., Milwaukee meeting, August, 1947.
† This is a preliminary report. Work is to be continued using the fresh plant when available.

EXPERIMENTAL

The leaves were collected from flowering plants in Greer County, Oklahoma, in June, 1946. They were carefully washed and spread out to dry in the shade. The air-dried material was reduced to a No. 20 powder in a Wiley mill.

Extraction with Selective Solvents.—Using two samples in Soxhlet Extractors and the solvents in the order listed, the following results were obtained:

Solvent	Per Cent Extractive
Petroleum ether (b. p. 30–60°)	0.898
Ether	0.639
Chloroform	1.78
Alcohol (dehydrated)	15.40
Solvent containing 50% alcohol and 50% water	4.41

Petroleum Ether Extract.—The petroleum ether extractive was redissolved in the solvent in order to separate a white substance that had appeared in the evaporation of the original extract. This was separated by filtration and dissolved in hot alcohol. Upon evaporation of the alcohol, a residue was obtained that was somewhat crystalline, but not uniformly so. Carbon tetrachloride was used in an attempt to obtain a better crystalline product but without success. A small amount of the material was heated to a temperature of 175° in a tube at 1-mm. pressure but none of it sublimed. A melting point determination gave an indefinite melting point of approximately 268°.

Alcohol Extract.—During the alcohol extraction, crystals were observed in the alcoholic extract. These crystals were separated and identified as potassium nitrate by the standard qualitative tests. All subsequent extractions with alcohol or water gave a yield of this salt that interfered with the attempts to purify other constituents. A nitrate determination was made on the aqueous extract from an air-dried sample using a method described by Klein (2). This showed the air-dried leaves to contain 2.4% of potassium nitrate.

The alcoholic extract was treated with hot water to obtain the water soluble fraction. The aqueous solution was tested with Benedict's qualitative solution. There was no reaction. After a small amount of hydrochloric acid was added, and the solution warmed, an insoluble substance precipitated. After filtration, the filtrate gave a positive test with Benedict's reagent. This showed the presence of a glycoside. Its formula was not determined other than to establish the identity of the sugar as glucose by its optical activity and the formation of an osazone.

Chloroform Extract.—The chloroform extract was redissolved in chloroform and extracted with dilute aqueous sulfuric acid. The aqueous acid solution was made alkaline with ammonia and extracted with chloroform. This extraction process was repeated. The evaporation of the chloroform gave a very small amount of a white residue that was insoluble in water but soluble in chloroform and carbon tetrachloride.

In order to obtain more of this material, 100 Gm. of the powdered leaves were mixed with a menstruum of two volumes of chloroform and one volume of ether and then 10 cc. of ammonium hydroxide was added. The sample was allowed to macerate overnight and then extracted with chloroform using a Soxhlet extractor. The chloroform extract was then treated as before, except that the solution was used without evaporation. The residue obtained on the final evaporation represented 0.038% of the sample. The material contained a small amount of an oil-like substance that had a strong unpleasant odor. This was removed by washing with ether. The material, at this point, gave a melting point of 135–138°. The white residue, dissolved in 1% hydrochloric acid, gave a precipitate with the following alkaloidal reagents; Mayer's, phosphotungstic acid, and gold chloride. Negative tests were obtained with platinic chloride, ferric chloride, and picric acid.

Since the plant is in the same family as belladonna, it was thought possible that the substance might be atropine but Vitalli's test was negative. A purple color was obtained, however, by omitting the preliminary treatment with nitric acid. A similar color was obtained by using 90% sulfuric acid.

Biological Tests.—In order to determine which was the active portion of the plant, approximately 500 flies (*Musca domestica*) were divided into three test groups. Using diluted skim milk as the vehicle, the flies in one group were given the impure glycosidal material, another group was given about 4 mg. of the alkaloid and the third group was used as a control. After four hours the flies getting the glycoside were dead, and the other groups were not affected. These were allowed to continue overnight but they were still unaffected the next morning. The control group was then given the skim milk mixed with the powdered leaves. This proved fatal to all of them. The group having the alkaloid remained unaffected. This showed the glycosidal material to be toxic while the alkaloid was nontoxic in the small dosage given.

SUMMARY

Physalis mollis Nuttall, is a plant that is indigenous to Oklahoma and has been used as a fly poison by residents of that state. Its toxic effect on the common house fly was demonstrated.

A glycoside was isolated in an impure form. This was found to be toxic to flies (*Musca domestica*). The glycone of the glycoside was identified as glucose. An alkaloid was isolated that showed some characteristic tests. It has a melting point of 135–138°. This substance was not toxic to flies in the small dosage used.

Potassium nitrate was found to be present in the amount of 2.4 per cent in the air dried leaves.

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Chemical Determination of Synthetic Estrogens. II. Application to Pharmaceutical Preparations of Diethylstilbestrol*

By SIDNEY GOTTLIEB

Methods are presented for the analysis of tablets, oil solutions, and cosmetic creams containing diethylstilbestrol by use of the nitrosophenol quinoid analysis previously reported.

In the previous paper (1) a method was proposed for the determination of a group of synthetic estrogens, consisting of nitrosation of the estrogen to the *o*-nitrosophenol and measurement of the intense color of the quinoid form of the *o*-nitrosophenol in alkaline solution. Data were presented for diethylstilbestrol, hexestrol, dienestrol, benzestrol, and meprane. It is the purpose of this paper to report on methods which have been developed to apply this reaction to various pharmaceutical preparations of diethylstilbestrol.

The U. S. P. XIII lists Diethylstilbestrol Tablets, Diethylstilbestrol Injection, and Diethylstilbestrol Capsules. The two latter forms are solutions of the estrogen in oil. Nonofficial forms of synthetic estrogens are in wide use. Creams, both vanishing and cold cream bases, containing synthetic estrogens, are examples of such preparations. Gynecological creams containing synthetic estrogens have also appeared on the market. Proposed methods for the analysis of such diethylstilbestrol preparations are described below under the headings Tablets, Oil Solutions, Cold Creams, and Vanishing Creams.

Materials

Buffer solution. To 800 cc. of glacial acetic acid add 150 cc. of 10% potassium hydroxide solution and 50 cc. of water, and mix well.

Saturated aqueous sodium nitrite solution.

Concentrated sulfuric acid (36 *N*).

Concentrated hydrochloric acid (12 *N*).

Alcoholic ammonium hydroxide solution. Mix 450 cc. anhydrous ethyl alcohol or isopropyl alcohol, 300 cc. of concentrated ammonium hydroxide (14 *N*), and 250 cc. of water.

Sodium hydroxide solution (1 *N*).

Sulfuric acid solution, 10%.

Alumina—activated alumina, 80–200 mesh.[1]

Silicic acid, C. P., precipitated.[1]

Isopropyl ether. Free 1 L. of the commercial chemical from peroxides by shaking with a solution made up from 150 Gm. of ferrous sulfate, 15 cc. of concentrated sulfuric acid, and 275 cc. of water. Following this treatment, wash the ether several times with distilled water and dry over anhydrous calcium chloride. It may be stored over calcium chloride and filtered before use. Do not use any ether that has been standing for more than a week.

Toluene, C. P.

Ethyl alcohol, 95%.

Tablets

Weigh accurately a portion of the powdered tablets equivalent to 15–20 mg. of diethylstilbestrol. Suspend the powder in a separatory funnel in 30 cc. of distilled water to which 5 drops of concentrated hydrochloric acid have been added. Extract this suspension twice with 25-cc. portions and three times with 10-cc. portions of peroxide-free isopropyl ether. Extract the combined isopropyl ether extracts first with 25 cc., then three times with 15-cc. portions of sodium hydroxide solution. Render the combined alkali extracts acid with dilute sulfuric acid and extract twice with 25-cc. portions and three times with 10-cc. portions of isopropyl ether. Filter the combined extracts through a dry filter and wash the filter with several small portions of isopropyl ether. Evaporate the ether to dryness and dissolve the residue in exactly 50 cc. of ethyl alcohol.

Take an aliquot of the final alcoholic solution equivalent to from 0.1 to 0.5 mg. of diethylstilbestrol and evaporate to dryness on the steam bath using a stream of air to remove the last traces of alcohol. Dissolve the residue in 5 cc. of buffer solution. Add 5 drops of concentrated sulfuric acid and 2 drops of saturated aqueous sodium nitrite solution. Allow this solution to stand thirty minutes with occasional mixing, then dilute to near 50 cc. with alcoholic ammonium hydroxide, cooling the volumetric flask in an ice bath as the alkali is added. Let stand thirty minutes, dilute to exactly 50 cc. with alcoholic ammonium hydroxide and filter a portion of this solution through a dry filter. De-

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[1] In this study, the alumina used was grade F-20 made by the Aluminum Ore Company, and the silicic acid was from Eimer and Amend, lot No. 403320. It should be emphasized that each lot of alumina and silicic acid used should first be standardized by passing a solution of the estrogen of known strength over a column of the adsorbent to determine whether or not complete recovery can be attained.

termine the amount of diethylstilbestrol in this filtrate by measuring its absorption at 440 mμ and comparing with a standard similarly treated, containing 0.2–0.3 mg. diethylstilbestrol, or by using a standard curve over the range 0.1 to 1.0 mg. diethylstilbestrol.

Oil Solutions

This method can be used for both oil-containing capsules and oil for injection in ampuls. The U. S. P. XIII specifications for removing the oil sample may be applied, using a sample containing about 5 mg. diethylstilbestrol, and dissolving the oil in 50 cc. of isopropyl ether. Proceed as described under "Tablets" above, beginning with "Extract the combined isopropyl ether extracts first with 25 cc., then three times with 15-cc. portions of sodium hydroxide solution."

Creams and Ointments

Under this heading are included vanishing and cold cream bases for skin application, and also various types of gynecological creams. It was necessary to develop separate methods for the cold cream base preparations (high oil/water ratio) and for the vanishing cream base (low oil/water ratio).

a. Cold Cream Base.—Accurately weigh an amount of cream containing 2–5 mg. of diethylstilbestrol. Transfer with water and toluene to a 500-cc. flask, using a total of 100 cc. toluene and 50 cc. of water. Add 1 cc. of concentrated hydrochloric acid, and reflux the mixture until a clear separation of the two layers is attained. This usually takes from twenty to forty minutes of moderate refluxing. Carefully separate the layers and shake the aqueous layer with three 25-cc. portions of toluene, and combine the toluene extracts with the original toluene layer. Prepare a 15- x 160-mm. adsorption column of activated alumina and wet with toluene. Pass the toluene solution through the column, followed in succession by 100 cc. of toluene and 100 cc. of isopropyl ether. Then run 100 cc. of 95% ethyl alcohol through the column, collect the alcohol and evaporate it to dryness. Dissolve the residue in 50 cc. of isopropyl ether and continue the analysis as described under "Tablets," beginning with "Extract the combined isopropyl ether extracts first with 25 cc., then three times with 15-cc. portions of sodium hydroxide solution."

b. Vanishing Cream Base.—Use the size of sample and the separation of the emulsion into an aqueous and a toluene phase as described under "Cold Cream Base." Use the same amount of concentrated hydrochloric acid. Pass the combined toluene extracts over a column prepared by intimately grinding 10 cc. methyl alcohol into 20 Gm. of anhydrous precipitated silicic acid. Make this mixture into a slurry with toluene and pour into a column 32 mm. wide. After the slurry runs nearly dry, put on the combined toluene extracts, followed in succession by 100 cc. of toluene and 200 cc. of isopropyl ether. Continue the analysis, using the 200-cc. isopropyl ether filtrate, as described under "Tablets," beginning with "Extract the combined isopropyl ether extracts first with 25 cc., then three times with 15-cc. portions of sodium hydroxide solution."

RESULTS AND DISCUSSION

Table I indicates the results of the analyses of several different preparations, both commercial samples and laboratory preparations.

The laboratory mixture of tablet powder base was made up of 90% corn starch, 3% $CaCO_3$, 3% talc, 2% lactose, and 2% sucrose. Diethylstilbestrol was thoroughly mixed with the powder so that it was 0.095% of the total. In general, no difficulties were encountered with tablet preparations.

The laboratory mixture of oil solution consisted of 0.1% diethylstilbestrol in a mixture of equal parts of U. S. P. Corn Oil, Cottonseed Oil, and Sesame Oil. In preliminary work, an attempt had been made to include a chromatographic step, using an alumina column, since it was felt that the steroids from the oils would appear in the final residues for analysis. It was found that the diethylstilbestrol could be adsorbed from dry isopropyl ether onto alumina by washing the column with isopropyl ether and isopropyl ether-ethyl alcohol mixtures and the estrogen could finally be washed off in fairly pure state with 95% ethyl alcohol. However, since sterols in moderate amounts do not interfere with the nitrosophenol analysis, and since it was difficult to standardize different batches of alumina, this step was abandoned. It has been used occasionally in special cases where an interfering substance makes it necessary.

The cold cream laboratory mixture was made by melting together 350 Gm. lanolin, 350 Gm. mineral oil, 84 Gm. spermaceti, and 98 Gm. of beeswax at 60°. Seven-tenths gram naphthylamine, 1 Gm. β-ionone, 0.7 Gm. ethyl-*p*-hydroxybenzoate, and 100 mg. diethylstilbestrol were stirred into this melt and the whole was heated to 70°. Seven grams of borax, dissolved in 511 Gm. of water at 72°, was added to the other solution with constant stirring allowing the temperature to fall during the mixing to 45–50°, at which temperature it was bottled. It will be noted that the esters of *p*-hydroxy benzoic acid, which are common preservatives for cosmetic creams, do not interfere with the analysis, although a free phenolic group is present. When conventional shakeout procedures were used, it was found that these preservatives would interfere with the analysis, and also that very troublesome emulsions would be formed in the initial shakeouts. The refluxing with toluene affords a simple method of cleanly breaking the emulsion, and the subsequent adsorption step allowed essentially everything but the estrogen to be separated from the column. Particular attention was given to the cold creams, because the great majority of estrogen creams appearing on the market are of this type. This type of separation could readily be applied in other cases where a

TABLE I.—DIETHYLSTILBESTROL ANALYSES ON VARIOUS PHARMACEUTICAL PREPARATIONS

Dosage Form	Sample	Amount of Stilbestrol Known or Declared, Mg.	% of Stilbestrol Known or Declared	Amount of Stilbestrol Found, Mg.	% of Known or Declared Amount Found
Tablets	1. Laboratory mixture	4.80	0.095 (*W/W*)	4.73	98.6
	2. Replicate of 1	4.80	0.095 (*W/W*)	4.67	97.2
	3. Replicate of 1	4.80	0.095 (*W/W*)	4.70	98.0
	4. Comm. sample—1 mg./tab.	15.00	2.5 (*W/W*)	14.86	99.0
	5. Comm. sample—0.5 mg./tab.	20.00	0.29 (*W/W*)	19.61	98.1
Oil Solutions	1. Laboratory mixture	4.68	0.1 (*W/V*)	4.62	98.8
	2. Replicate of 1	4.68	0.1 (*W/V*)	4.66	99.6
	3. Replicate of 1	4.68	0.1 (*W/V*)	4.64	99.2
	4. Capsules, 5 mg./cap.	25.00	... (*W/V*)	24.32	97.4
	5. Oil for injection, 5 mg./cc.	5.00	0.5 (*W/V*)	4.93	98.6
Cold Creams	1. Laboratory mixture	2.00	0.071 (*W/W*)	1.94	97.0
	2. Replicate of 1	2.00	0.071 (*W/W*)	1.96	98.1
	3. Replicate of 1	2.00	0.071 (*W/W*)	1.93	96.6
	4. Comm. sample—2.5 mg./oz.	2.50	0.089 (*W/W*)	2.42	96.8
	5. Comm. sample—2.5 mg./oz.	2.50	0.089 (*W/W*)	2.45	98.0
Vanishing Creams	1. Laboratory mixture	4.00	0.143 (*W/W*)	3.91	97.8
	2. Replicate of 1	4.00	0.143 (*W/W*)	3.89	97.3
	3. Replicate of 1	4.00	0.143 (*W/W*)	3.93	98.2
	4. Comm. sample—2 mg./oz.	2.00	0.071 (*W/W*)	1.96	98.0
	5. Comm. sample—2.5 mg./oz.	2.50	0.089 (*W/W*)	2.39	95.6

minute amount of a substance to be determined is incorporated in a large amount of cream.

The laboratory preparation of vanishing cream was carried out by adding a solution of 98 Gm. of glycerin, 9.24 Gm. of potassium hydroxide, and 5.6 Gm. of triethanolamine in 700 cc. of water heated to 85° to a solution of 200 mg. stilbestrol in 185 Gm of stearic acid heated to 85°. This addition was carried out under constant and vigorous stirring. When this mixture had cooled to 50°, a dispersion of 28 Gm. of glyceryl monostearate, 2 Gm. of β-ionone, 0.7 Gm. of butyl-*p*-hydroxy benzoate in 374 Gm. of water was thoroughly incorporated and the mixture cooled and bottled. When the method designed for the cold cream base was applied to this preparation, very low recoveries were obtained, apparently due to the solubility of the estrogen in the large amount of stearic acid. In every method tried for separating the stearic acid from the estrogen, the stearic acid carried along appreciable amounts of stilbestrol. Several adsorbents, including talc, sucrose, starch, and celite, were ineffective. The method finally adopted utilized the partition chromatographic technique originally discussed by Martin and Synge (2). The method is not entirely suitable for routine analysis, since the preparation of the column entails considerable time and care, and since different batches of anhydrous precipitated silicic acid will vary greatly and hence must be carefully standardized for the particular separation contemplated. The method is reported here, however, because it does give good results when carefully used.

Preliminary experiments indicate that dienestrol, benzestrol, hexestrol, monomestrol, and meprane can be readily determined in tablet and oil preparations by the method outlined above. The meprane must first be hydrolyzed to the free phenol. Their determination in the cosmetic and gynecological creams is yet to be made. It is to be expected that the structural variations in these compounds would bring about differences in their behavior on the adsorbents utilized in the analysis of creams. However, a determination of a gynecological cream containing dienestrol has been successfully carried out by the method outlined for cold creams.

SUMMARY

Methods are presented for the analysis of diethylstilbestrol in tablets, oil solutions, and creams, utilizing the formation of nitrosophenol quinoid compounds. Results indicate the method can be successfully applied to these preparations.

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A Photoelectric Colorimetric Assay for Gentian Violet by Comparison with a Standard Powder*,†

By JOSEPH D. MC INTYRE‡ and ARTHUR E. JAMES

A colorimetric photoelectric quantitative method has been developed whereby the gentian violet content of tablets may be determined by comparison with a standard reference powder. Samples of gentian violet may be compared with each other.

THE official pharmaceutical compendiums in the United States and in several foreign countries are reflecting the growing importance and acceptance of gentian violet. Since 1925 New and Nonofficial Remedies (1) has included it as an acceptable therapeutic agent. The Swiss Pharmacopœia of 1933 (2) listed gentian violet under the title of "Methylrosaniline Chloratum." It was introduced into the National Formulary VI (3), in 1936, under the title of "Methyl-rosaniline," using as synonyms the terms Gentian Violet, Methyl Violet, and Crystal Violet. The National Formulary VII (4) carried Methyl-rosaniline Chloride solution while the current edition, National Formulary VIII (5), continues to list the solution and has added a jelly. The United States Pharmacopœia XII, issued in 1940, (6) adopted Methyl Rosaniline Chloride. The Extra Pharmacopœia of Great Britain (7) adopted Medicinal Gentian Violet in 1936 under the title of Methyl Violet. Notwithstanding the increased use of gentian violet none of the official reference books contains directions for its assay.

Albert (8) has recently suggested a gravimetric method for the assay of gentian violet in which a suitable weight of sample dissolved in water is heated with 1 *N* NaOH. The resulting insoluble carbinol is extracted with benzene. After filtering, the benzene extract is evaporated and the residue is dried to constant weight. Each gram of carbinol is equivalent to a fixed weight of gentian violet. However, the Albert procedure has proved unsatisfactory in the hands of competent analysts when applied to the assay of tablets of gentian violet. The carbinol is so tenaciously adsorbed on the tablet excipients that it is very difficult to extract. Conn (9) and others (10) have suggested a volumetric assay for gentian violet using a standard solution of titanous chloride, $TiCl_3$. Titration with this chemical has several limitations including the necessity of excluding air from the solution.

In view of the intense color of aqueous solutions of gentian violet and of the general acceptance of photoelectric colorimetric methods for rapid analysis this type of assay has been studied in our laboratories and has been found to give satisfactory results.

EXPERIMENTAL[1]

Preparation of a Standing Graph for Comparison.—A sample of U. S. P. gentian violet[2] was dried to constant weight in a vacuum desiccator over concentrated sulfuric acid. Exactly 0.5 Gm. of this powder was dissolved in distilled water and diluted to a liter. Separate dilutions of this solution containing from 0.50 to 5.0 mg. of gentian violet per liter were prepared. Experiment indicated that the maximum absorption of U. S. P. gentian violet solutions is at 586 mμ. A light filter with maximum transmission between 500 and 570 mμ was used. With a Klett–Summerson photoelectric colorimeter[3] the transmission of the various solutions was determined and read on the logarithmic scale of the instrument. A typical set of data is as follows:

Concentration of Gentian Violet, Mg./L.	Colorimeter Reading
5.08	320
4.50	286
4.00	254
3.50	224
3.00	194
2.50	160
2.00	128
1.66	107
1.25	79
1.00	65
0.50	32

* Received Oct. 7, 1947, from the Research Laboratories of Dr. D. Jayne & Son Branch, Sterling Drug, Inc., and Temple University School of Pharmacy, Philadelphia.

† The powder accepted as standard in this investigation was supplied by the National Aniline Division of the Allied Chemical & Dye Corporation, Buffalo, N. Y. It is suggested that the U. S. P. Revision Committee establish a standard reference powder.

‡ Deceased Jan. 25, 1948.

[1] Acknowledgment is made to Joseph Marlino of Dr. D. Jayne & Son Branch, Sterling Drug, Inc., for checking experimental data shown herewith.

[2] See footnote indicated by dagger (†)

[3] Any suitable photoelectric colorimeter may be used.

The above data are transcribed to graphical form in Fig. 1. The concentration of gentian violet is plotted as abscissa and the corresponding colorimetric readings as ordinate.

Assay of Individual Tablets Containing Gentian Violet.—Individual enteric coated tablets claimed to contain approximately 25 mg. of gentian violet were assayed as follows. The outer sugar coating and enteric coating were removed by solution in distilled water. The nucleus of the tablet was pulverized after drying and the resulting powder dissolved in distilled water and made up to a volume of 1 L. Suitable dilutions were made to bring the concentration within the range of the colorimeter scale. The transmission of each solution was measured in the colorimeter and the corresponding concentrations of gentian violet interpolated from the standard graph. Typical results were as follows:

Colorimeter Reading	Gentian Violet, Mg.
158	24.70
157	24.65
147	23.00
156	24.67
153	24.00
143	22.30
158	24.70
151	23.50
150	23.50
144	22.30
	Av. 23.73 mg./tablet

Assay of Lots of 20 Tablets of Gentian Violet.—After suitable preparation and dilution the gentian violet content of each lot was determined colorimetrically. Typical data obtained were as follows:

Manufacturer or Source of Tablets	Labelled Content of Gentian Violet/Tablet, Mg.	Amount of Gentian Violet Found/Tablet, Mg.
A for adults	25	23.5, 23.4, 22.6
B for adults	25	23.5, 23.5, 22.6
A for children	9.7	8.5, 8.5, 8.0
C for children	9.7	8.5, 8.6, 8.3
A uncoated tablet nuclei		27.5, 28.0, 28.5

DISCUSSION OF RESULTS

It was found expedient to dry gentian violet to constant weight in a vacuum desiccator over concentrated sulfuric acid. Efforts to dry it by heating in an oven at 110°, as specified in the U. S. P., required several weeks before attaining constant weight. The reason for this appears to be due to the very gradual loss of water of hydration. Several hydrates—a tri, and hexa or nona hydrate—of crystal violet have been reported (8).

The calibration graph as shown in Fig. 1 shows close adherence to Beer's Law indicating that the color intensity is in direct proportion to the concentration of gentian violet.

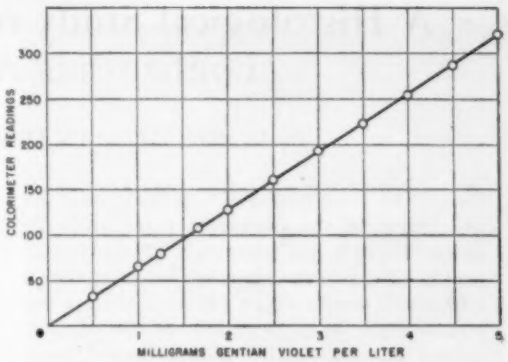

Fig. 1.—House Reference Standard. Dried to constant weight.

Aqueous solutions of gentian violet were found to be stable when exposed to daylight for several months. Solutions of tablets made by some manufacturers were not stable toward light if exposed for several hours. However, the solutions of tablets obtained from other manufacturers were stable. This difference in behavior is attributed to differences in tablet excipients.

It is noteworthy that, with the exception of the uncoated tablet nuclei, the results of the above assays show less gentian violet than the labelled content. It is believed that this is due to the presence of small amounts of reducing sugars which are present in both the enteric coating and in the excipient of the tablets.

For laboratory control of manufacturing processes, it is desirable that a rapid assay method to determine the content of gentian violet be developed. The colorimetric assay proposed herewith fulfills this need. It is applicable to the assay of samples of gentian violet and to the assay of tablets containing this product. Either single tablets or lots of 20 may be used.

As the U. S. P. does not at present have an assay method to determine the potency of gentian violet the authors of this paper believe that it would be in the interest of public health for the Revision Committee to establish a standard reference powder.

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A Histological Study of the Glandular Hairs of Lophanthus Anisatus Benth.*

By ROBERT L. VAN HORNE† and LOUIS C. ZOPF‡

Results of a study of the morphological development of the glandular hairs of *Lophanthus anisatus* are presented. Microphotographs illustrate the steps in the cell division which lead from a single epidermal cell to the mature eight or more celled volatile oil-producing glandular hair. Tissue tests demonstrate that cutin and pectic materials are present in the outer wall of the glandular hairs.

THE cultivation of mint plants as farm crops has been practiced in the United States, as well as abroad, for many years. The United States Department of Agriculture has carried on intensive investigations regarding growing conditions, methods of harvesting the plants, and the problems attendant to extracting the volatile oils. Van Fleet (1), and Sievers (2), have studied thoroughly the effects of various cultivating conditions on the peppermint plant which is recommended as a profitable farm crop.

More recently, several workers have investigated mint plants from the standpoint of the chemical composition and possible therapeutic use of their volatile oils. In the course of this work, attention was directed to the location and structure of the cells producing the volatile oils. Hocking and Edwards (3) attempted to correlate the numbers and dimensions of the glandular scales of *Mentha piperita* with the amount of oil produced in the species. Some interesting comparisons of the volume of oil produced by species of the plants grown in Florida and in Indiana resulted, but little correlation was found to exist between the number of oil cells and the volume of oil produced.

Ripert (4), in a discussion of the location of the oil-producing tissues of peppermint, stated that the volatile oil develops in the protoplasm of the epidermal foot cell, and that this cell is the parent cell of the glandular scales. He concluded that the oil is a waste product of metabolism.

Murthy (5), of the Department of Botany, Central College, Bangalore, India, studied the development of the glandular hairs of *Orthosiphon stamineus*, another member of the *Labiatae*. He illustrated, by drawings, how the hair first appears as an enlarged epidermal cell which divides periclinally to form a stalk cell and a head cell. The stalk cell then divides periclinally again to form a middle cell and a basal cell. The head cell divides anticlinally into two cells. These cells divide further into four cells and again into eight. The volatile oil is secreted beneath the cuticle which is distended to form a bladder which ruptures when the pressure of the oil becomes too great for the cuticle to withstand, thus liberating the oil into the atmosphere.

Recently, Politis (6) published his observations regarding the secretion of essential oils in plants belonging to the *Labiatae* and other volatile oil-producing plants. He asserts that the cuticle on the glandular hairs raises to form the balloon-shaped pocket into which the essential oil is secreted.

This investigation of *Lophanthus anisatus* was undertaken to show by the use of photomicrographs and drawings the sequence of development of the glandular hairs.

The selection of the subject plant was influenced by the fact that it was already growing in the Pharmacy-Botany gardens of the State University of Iowa. It had been planted there from seed furnished in 1942 by Mr. Frank Pellett, of the Pellett horticultural gardens of Atlantic, Iowa. Pellett had published several papers on the usefulness of the plant as an important source of nectar for honey bees (7–9).

Lophanthus anisatus Bentham, commonly

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† Fellow, American Foundation for Pharmaceutical Education.
‡ Professor of Pharmacy, College of Pharmacy, State University of Iowa.

called anise hyssop because of its fragrant anise-like odor, is a slender, tall, herbaceous plant which may reach a height of one meter or more, Fig. 1. It is known by several other botanical synonyms, *Agastache anethiodora* Nutt. and *Agastache foeniculum* Pursh being the more common of them. In addition to the common name of anise hyssop, it is also called *Hyssopus ambiguous* but is not to be confused with *Hyssopus officinalis* which is the well-known cultivated variety of the hyssop. Its habitat ranges from Illinois westward to the foothills of the Rocky Mountains in Colorado and northward to Manitoba.

The plant in bloom is characterized by its fragrant spike of blue flowers which grow in a dense head and attract all sorts of insects from the moment the flowers open until blooming ceases in late summer.

Fig. 1.—*Lophanthus anisatus*, Benth.

It produces a volatile oil which has been analyzed by Polak and Hixon (10) recently and which was also reported on in 1898 and 1913 by Schimmel and Company (11, 12). We did not attempt an analysis of the oil in our work.

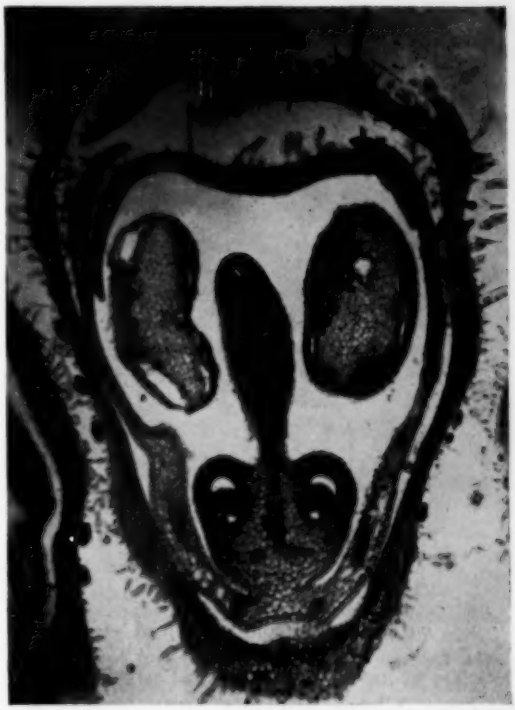

Fig. 2.—Longitudinal section through flower bud. All three types of hairs are visible on outer surface of calyx.

EXPERIMENTAL

Since we wished to trace the stages in the growth and development of the glandular hairs, we collected specimens at intervals allowing for various stages of maturity. The first collections were made on April 7, 1946, and collections of the upper leaves were made at intervals of about two weeks until the first week of June when the flowers opened. As soon as the material was collected, it was killed and fixed in a formalin–acetic acid–alcohol mixture. This was found to be a good killing fluid for leaves but the flowers, because of their succulent nature, were more satisfactorily fixed in a modified Nawaschin solution. Paraffin was used to embed the material for sectioning on the rotary microtome.

Safranin and fast green were employed to stain the sections preparatory to examining them for the glandular hairs. These stains gave good contrast in the photomicrographs. During the process of staining the tissues we became interested in determining, if possible, the nature of the material which forms the balloon-like sacs about the mature glandular hairs. As mentioned before, Politis stated that the cuticle about the hairs becomes distended into the balloon-like shape when the cells secrete their volatile oil.

Using prepared sections from killed and fixed material, we tried a number of tests to determine whether the material composing the outer wall is really cutin or a mixture of substances such as pectic

Fig. 3.—Mature glandular hair with collapsed cuticle. Nonglandular hair at left.

materials, cellulose, and cutin. By immersing sections of the flower heads, which had numerous glandular hairs on the corolla and calyx, in 50% chromic acid solution in the cold, all the material but the envelope surrounding the glandular hair, and the cuticle of the epidermal layer, was dissolved away. Upon staining the residue left on the slide with Sudan IV, a red color was produced in the cuticle of the epidermis and also in the sacs which had enveloped the hairs, indicating the probability that at least some of the material in the sac is cutin. It is not reasonable to expect that cutin alone could expand or stretch sufficiently to maintain its intact character as the glandular hairs grow from the one-celled stage to maturity. By the use of Ruthenium Red in a very dilute aqueous solution, we were successful in demonstrating the presence of pectic materials in freshly cut sections bearing glandular hairs. It is apparent then that the sac surrounding each glandular hair is composed of both cutin and pectic material.

DISCUSSION

Three types of hairs occur on the leaves and flower parts of *Lophanthus anisatus*. Most numerous is the nonglandular, papillose, several-celled type of hair. These are abundant on the lower epidermis of the leaf and also on the outer surfaces of the corolla and calyx, Fig. 2. The number of cells varies from one to three, or rarely, four. The apical cell is almost always sharply pointed. In many of these hairs, the nucleus is visible in the young plant, and stains red with rapid safranin. These hairs are usually straight but may recurve somewhat, particularly those found on the lower leaf surfaces.

A second type of hair is composed of a basal cell, a stalk cell, and one or two glandular head cells. A prominent cuticle surrounds the head cells and they stain deeply with safranin in the flowering plant, but are unstained in the plants which have not yet reached the stage of anthesis. They occur in lesser numbers than the nonglandular hairs but in the same areas. In some cases they may be found in scattered points on the upper epidermis of the leaf but their most common location is on the lower leaf surfaces and on the outer surfaces of the calyx and corolla. These one- or two-celled glandular hairs are present in all stages of formation of the third type of hair, the multicelled, balloon-shaped glandular hairs, and appear to be distinct from the latter Fig. 3.

This type is supported by a unicellular stalk and arises from a large epidermal cell surrounded by a number of much smaller epidermal cells. The ontogeny of the glandular hairs is the main subject of this paper.

As observed in the very young leaf and flower, the development of the typical multicellular glandular hair begins with the enlargement of an epidermal cell. Such an epidermal cell in the young tissue has not yet developed a distinct cuticle and its outer

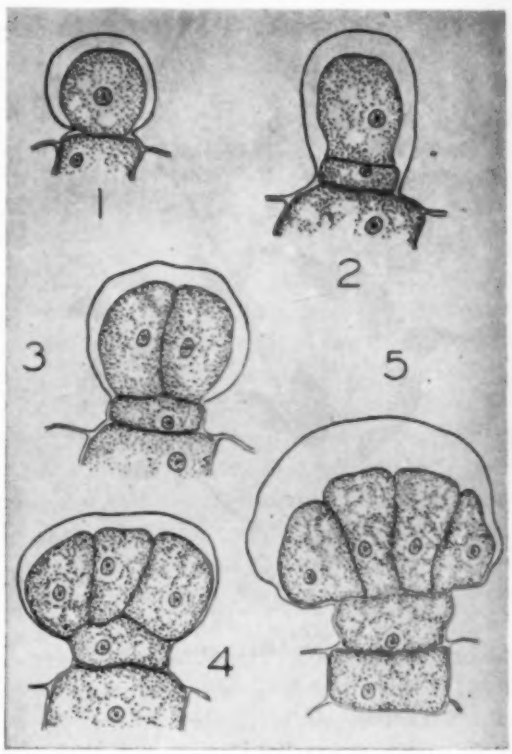

Fig. 4.—Drawings of various stages of development of glandular hairs. *1*—First stage after initial division of epidermal cell. *2*—Head cell differentiated from stalk cell. *3*, *4*—Successive stages in cell division in head results in mature glandular hair with eight or more secreting cells—*5*.

wall is still elastic. The increase in cell contents stretches the upper wall so that the enlargement of the cell is in a direction external to the epidermal layer. Subsequent division of the enlarged epidermal cell gives rise to a daughter cell, Fig. 4. Upon completion of the division of the epidermal cell and the subsequent enlargement of the daughter cell, a periclinal division of the new cell occurs and the result is the head cell and the stalk cell, which latter retains its identity throughout the remainder of the glandular hair formation. By anticlinal division, the head cell gives rise to two head cells, Fig. 5, and continued anticlinal division results in the formation of first, four head cells and then eight, with rarely, ten or twelve cells, Fig. 6, occurring in the mature glandular hair.

In this process of division, the cuticle surrounding the entire glandular hair enlarges so that the mature hair has a balloon-shaped cuticle intact around the whole of the head.

As the secretion of the volatile oil commences in the glandular hair, the cuticle becomes distended and may eventually burst, Fig. 3, releasing the volatile contents into the atmosphere (4, 13). Hanstein (14) states that the cuticle may be regenerated in some cases and the process of secretion may be repeated in the same glandular hair. Ripert (4) concurs in this conclusion.

Fig. 6.—Cross section through head of twelve-celled glandular hair. Stalk cell is out of plane of focus.

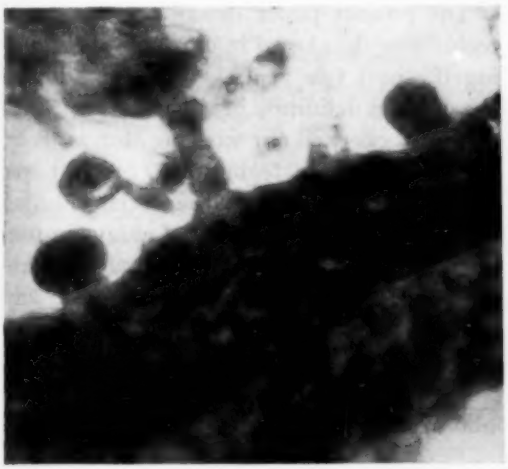

Fig. 5.—Two-celled glandular hair (right) and 4-celled hair (left) of *L. anisatus*. The stalk cell of each hair is also visible.

There are various opinions as to the reason for the formation of the oil and also as to the location of the initial secretion. Haberlandt (13) believed that the secretion was derived from raw materials present in the original cell contents, and in his later work Politis concluded that glycosides were the precursors of the essential oils. The fact that in staining glandular hairs in anise hyssop in the fresh condition with Sudan IV, numerous globules and granular inclusions in the head cells were observed, leads one to conclude that the oil may be formed in the cells of the glandular hair. Also, in dehydrated material, there remained granular material which stained deeply with safranin and made observation of the nuclei difficult. Such stained material made it impossible to obtain clear resolution of the individual cells for photomicrographs.

Photographs of the various phases of the cell division in the development of the glandular hairs were made to show clearly the steps in the process. It proved difficult to achieve clear-cut pictures due to the high magnification required and the accompanying lack of depth of focus. However, it is hoped that the pictures will aid in the clear understanding of the steps in the cell division.

SUMMARY

1. The development of the secretory cells which produce the volatile oil in *Lophanthus anisatus* has been observed and recorded by the use of photomicrographs.

2. The sequence of the development of the glandular hairs starts with the enlargement of an epidermal cell; this divides into a basal cell and a stalk cell. The stalk cell divides periclinally to give a head cell and a basal stalk cell. The head cell divides anticlinally into first two, then four, and finally eight cells. Some further development may

occur in a few hairs giving rise to as many as ten or twelve cells in the head. This development is therefore similar to that observed for a number of other species.

3. The composition of the cuticle surrounding the glandular hair was determined by tissue tests to be cutin, pectic materials, and possibly some other undetermined material.

4. The volatile oil is apparently secreted in the glandular hair causing the cuticle surrounding the hair to become distended with the accumulating secretions and to rupture.

5. Drawings to supplement the photographs depict the steps in the cell division in the glandular hairs.

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The Assay of Bacitracin*

By GRANT D. DARKER, HELEN B. BROWN, ALFRED H. FREE,† BARBARA BIRO, and JOHN T. GOORLEY

Two procedures, turbidimetric and cylinder plate, are reported for the assay of bacitracin. Two standard curves based on the Food and Drug Administration and the Cooper–Woodman methods are shown for the cylinder plate assay. The mathematical relationship between the antibiotic concentration and the zone of inhibition of bacitracin is discussed.

IN 1945 Johnson, Anker, and Meleney (1) described an antibiotic which they named bacitracin. It is produced by an organism of the *Bacillus subtilis* group which was isolated from a contaminated wound. A serial dilution method of assay was described in which a stock strain of a Group A hemolytic streptococcus was used as the test organism. A tentative standard unit was defined by this technique as "the amount which when diluted 1:1024 in a series of twofold dilutions in 2 cc. beef infusion broth, completely inhibits the growth of a stock strain of Group A hemolytic streptococcus when the inoculum used to seed the tubes is 0.1 cc. of a 10^{-2} dilution of an overnight culture in blood broth."

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The present paper describes other assay techniques developed in our laboratory during the past two years. We have adopted the original definition of a unit of bacitracin and have checked our working standards by the serial dilution technique. Since it was not convenient to use the serial dilution method for a large number of assays in routine testing, two techniques already in use for penicillin assay, the turbidimetric and cylinder plate procedures, were modified for the assay of bacitracin. The cylinder plate method has been used routinely in our laboratories.

THE TURBIDIMETRIC ASSAY

The turbidimetric technique is a direct application of the procedure we have used for penicillin (unpublished data) and similar to that described by McMahan (2). It is rapid and sensitive from 0.2 to 0.8 unit per ml. of the sample. It is accurate to within ±20%. *Staphylococcus aureus* (F. D. A. 209-P) is grown in Bacto-yeast broth. The retardation of growth of the test organism obtained with a series of dilutions of the assay sample is compared with that obtained with a standard bacitracin solution. The tubes are incubated in a water bath at 38° for four and one-half hours when growth is stopped by steaming. The turbidity is estimated

by determining the per cent transmittance in a Coleman Universal Spectrophotometer at a wave length of 600 mμ. The amount of antibiotic activity, as measured by the turbidity, is given by the difference (Ts − Tb), between the growth obtained with the bacitracin sample (Ts) and that obtained with the organism alone (Tb). This in turn is compared to the inhibition obtained with a standard solution of bacitracin. Strictly sterile technique need not be observed. A typical curve is given in Fig. 1.

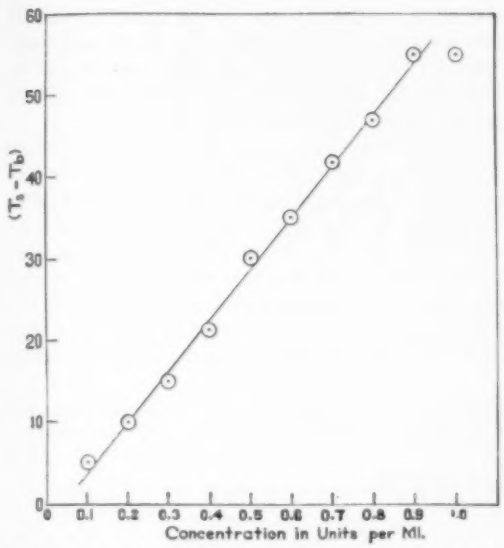

Fig. 1.—Turbidimetric assay. Ts − Tb *versus* bacitracin concentration.

Technique for Turbidimetric Method.—The assay as carried out in our laboratory is as follows: A suitable amount of sterile Bacto-yeast broth is inoculated with an 18-hour culture of *S. aureus*, 1 ml. per 100 ml. of broth. This is mixed thoroughly and dispensed into sterile test tubes in such amounts that the broth plus sample will be 10 ml. The standard solution is diluted with sterile uninoculated broth to a concentration of 1 unit per ml. The samples are diluted in the same way. These assay samples should have a *p*H between 4 and 6, contain no rapidly growing organisms, be clear, and have only slight color. There should be no organic solvents present as these will inhibit the growth of the bacteria. The inoculated broth is put into the tubes first. The diluted standard or samples are added by pipette, the tubes stoppered and immediately mixed. The series is set up so that from 0.1- to 1.0-ml. amounts are used in intervals of 0.1 ml. Blanks are made of uninoculated broth and control tubes of the inoculated broth alone. The racks of tubes are placed in a water bath at 38° for four and one-half hours. The racks of tubes are removed and steamed for ten minutes in the autoclave. The tubes are cooled, shaken, and read in the Coleman Spectrophotometer at a wave length of 600 mμ. The machine is set at 100% transmittance with the uninoculated broth blank. The difference between the per cent transmittance of the reading of any one tube minus the reading of the tube with *S. aureus* alone, (Ts − Tb), is a measure of the antibiotic concentration in that tube. The standard curve is run each day and the strength of the assay samples is taken from that curve.

THE CYLINDER PLATE ASSAY

The cylinder plate method is a modification of that employed for penicillin (3, 4). It is an adaption of the assay method of Schmidt and Moyer (4) in which the same organism, broth, agar, and buffer are used. It is suitable for solutions containing 1.5 to 8 units of bacitracin per ml. within an accuracy of ±15%. When the directions for the penicillin assay are followed precisely with the substitution of bacitracin for penicillin, small zones of inhibition are obtained and the variation of the zone diameters with variable concentrations of bacitracin is quite small. If the inoculated plates, with the cylinders holding the test solutions, are kept at 4° for six to ten hours prior to incubation, there is an increase in the zone size and greater variation in the zone diameter with varying concentrations of bacitracin. Refrigeration is necessary because the diffusion rate for bacitracin is lower than that for penicillin (see below).

Technique for Cylinder Plate Method.—The cylinder plate assay method is carried out in our laboratory as follows: An eighteen-hour culture of *S. aureus* (F. D. A. 209-P) is prepared in a yeast-beef broth. The frozen or dried bacitracin standard is diluted with 0.1 *M* phosphate buffer, *p*H 6.0, to give a working standard of 2.5 units per ml. All unknown samples are diluted with phosphate buffer to give a concentration of approximately 2.5 units per ml. When interfering substances such as alcohols are present in the samples, a dilution of at least 1:20 is necessary for accurate analyses. Plates are prepared by adding 22 ml. of nutrient agar to each, and are inoculated by flooding with 4 ml. of inoculated (0.8 ml. of eighteen-hour inoculum per 100 ml. melted agar) nutrient agar. The inoculated plates are kept at 4° until just before the cylinders are dropped in place. This prevents preliminary growth of the organism. Five cylinders are placed on each inoculated agar plate. Three of these are filled with the unknown sample and two with the standard. The plates are now refrigerated for nine to ten hours at 4° and then are placed in a 37° incubator for seven to eight hours. The plates are finally removed to the laboratory and the zones of inhibition are measured.

The Standard Curves.—The data for the standard curves were obtained from two samples of bacitracin which were standardized by the serial dilution method. In each test the working standard was diluted to give a range of solutions within the limits 0.5 and 40.0 units per ml. In all, seven series were

prepared, three with one standard and four with the other. On each plate two cups were filled with a 2.5 units per ml. solution and three with another concentration. Zones of inhibition were thus established for various concentrations of the standard in comparison with the 2.5 units per ml. solution. In tabulating the results, experimental values from plates showing the same zones of inhibition of the 2.5 units per ml. standard were segregated. Thus several series of data were established under the same experimental conditions against values of the inhibition zone of 2.5 units per ml. ranging from 15.0 to 18.0 mm. As the majority of the readings of the zones of inhibition of the 2.5 units per ml. concentration was in the 17.0 mm. series, this figure was selected for the basic standard curves. Table I shows two of the series with all of the experimental data for plates on which the 2.5 units per ml. standard gave zones of inhibition of 16.0 mm. (column 2) and 17.0 mm. (column 3), respectively. Column 1 gives the concentration in units per ml., and columns 4 and 5 record the numbers of readings on which columns 2 and 3, respectively, are based. The two columns to the right represent derived data used in the development of the Cooper–Woodman curve discussed later.

larger than that of the plate standard the difference is added to the zone size of the 2.5 units per ml. standard on the standard curve. This gives a corrected value for the unknown. For example, the unknown is 17.3 mm. and the plate standard is 16.5 mm., the difference, 0.8 mm., is added to 17.0 mm. to give a corrected value of 17.8 for the unknown. If the zone size for the unknown is smaller than that of the plate standard the difference is subtracted from the zone size of the 2.5 units per ml. standard on the standard curve. Thus, if the unknown is 15.3 mm. and the standard is 16.5 mm., the difference, 1.2 mm., is subtracted from 17.0 mm. to give a corrected value of 15.8 mm. for the unknown. Then for the corrected values of the zone size of the unknown the corresponding concentrations of bacitracin are read off the standard curve.

A series of standard curves has been plotted in our studies from experimental data assembled against readings of the 2.5 units per ml. standard ranging from 15.0 to 18.0 mm. All of these curves are essentially parallel. From a sufficiently large number of curves of this type it would be possible to read the value of the unknown without correction directly from the standard curve showing the same inhibition of the 2.5 units per ml. standard as the standard on

TABLE I.—RELATIONSHIP BETWEEN THE CONCENTRATION OF ANTIBIOTIC (m_0) AND THE ZONE OF INHIBITION ($2x + d$)[a] IN COMPARISON WITH 16.0- AND 17.0-MM. ZONES OF INHIBITION OF THE 2.5 U./ML. STANDARD

Units per Ml. (m_0)	Av. Zone of Inhibition ($2x + d$) against 2.5 U./Ml. Standard of 16.0 Mm.	17.0 Mm.	No. of Readings against 2.5 U./Ml. Standard of 16.0 Mm.	17.0 Mm.	Based on a 2.5 U./Ml. Standard Zone of 17.0 Mm. x	x^2
0.5	8.0	10.1	6	12	2.3	5.3
1.0	11.8	13.3	9	24	3.9	15.2
1.5	13.5	14.6	3	60	4.55	20.7
2.0	15.2	15.7	6	42	5.1	26.0
2.5	*16.0*	*17.0*	62	254	5.75	33.1
2.75	....	17.3	..	21	5.9	34.8
3.0	16.5	17.4	15	24	5.95	35.4
3.5	..	18.3	..	18	6.4	41.0
4.0	17.5	18.8	9	51	6.65	44.2
5.0	19.0	..	12	..	..	..
6.0	19.2	20.3	9	21	7.4	54.8
8.0	20.6	21.3	6	33	7.9	62.4
10.0	21.8	22.5	6	33	8.5	72.3
20.0	23.4	24.6	6	36	9.55	91.2
31.4	..	25.2	..	3	9.85	97.0
32.5	25.3	26.0	6	3	10.25	105.1

[a] d represents the inside diameter of the assay cylinder, in this example equal to 5.5 mm., and x represents the distance from the inner face of the cylinder to the edge of the zone of inhibition.

Figure 2 represents a standard curve of bacitracin determined according to the technique outlined by the Food and Drug Administration (5, p. 12133) for the assay of penicillin. It is based on the figures given in columns 1 and 3 of Table I for a standard zone of 17.0 mm. against a 2.5 units per ml. standard. In this graph on 3-cycle semilogarithmic paper, the diameter of the zone of inhibition has been plotted as the abscissa on the linear scale and the concentration of bacitracin as the ordinate on the logarithmic scale. To estimate the units in an unknown sample from a curve of this type the average zone sizes of the sample and the standard are calculated for the test plate. If the average zone size for the unknown sample is

the test plate. Since these standard curves on semilogarithmic paper are straight lines they represent the same type of variation expressed by the formula $y = a \cdot 10^{bF(x)}$. Log concentration or log $m_0 = \log a + b\,F(x)$, where $\log a$ represents the intercept on the y axis and b is the slope for any particular value of $F(x)$, in this instance $(2x + d)$. Therefore $\log m_0 - \log a \propto (2x + d)$. From this formula b may be determined for a known point and then corresponding values of m_0 may be calculated for various values of $(2x + d)$. Thus a chart may be prepared for direct reading of m_0 against any value of the zone of inhibition of the unknown solution. It is actually possible to construct a chart from two care-

fully determined points on a standard curve although in practice it is customary to establish a wide range of points in order to determine the useful limits of the curve.

Cooper and Woodman (6) have utilized the general formula in another form to express the relationship between the concentration of the antibiotic and the zone of inhibition in terms of physical constants.

$$\log m_0 = \frac{1}{4D\ 2.30} \cdot \frac{x^2}{t} + \log m'$$

where

D = the coefficient of diffusion of the solute
x = the distance from the inner face of the cup to the edge of the zone of inhibition
m_0 = the concentration of the antibiotic in the cup
m' = the smallest concentration of antibiotic showing inhibition on a solid medium
t = the critical time of growth beyond which the test organism shows no perceptible inhibition

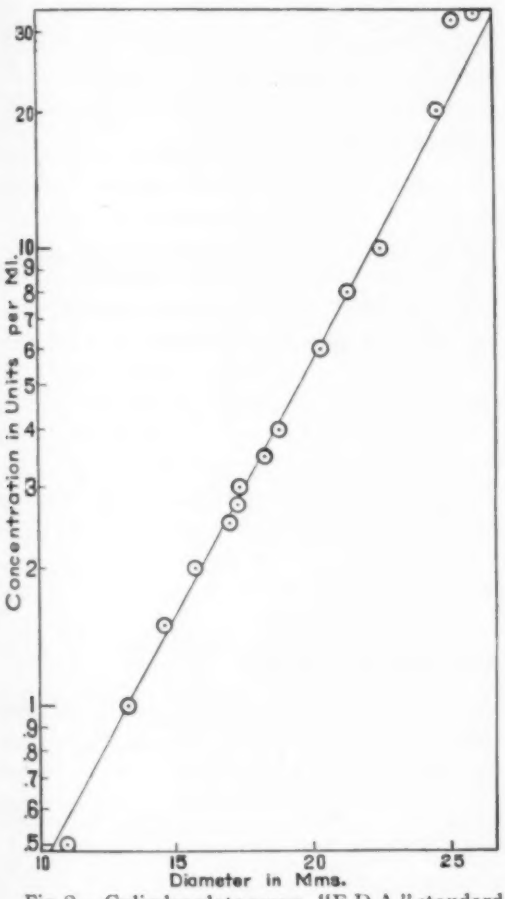

Fig. 2.—Cylinder plate assay. "F.D.A." standard curve. Zone diameters in mm. *versus* logarithms of bacitracin concentration.

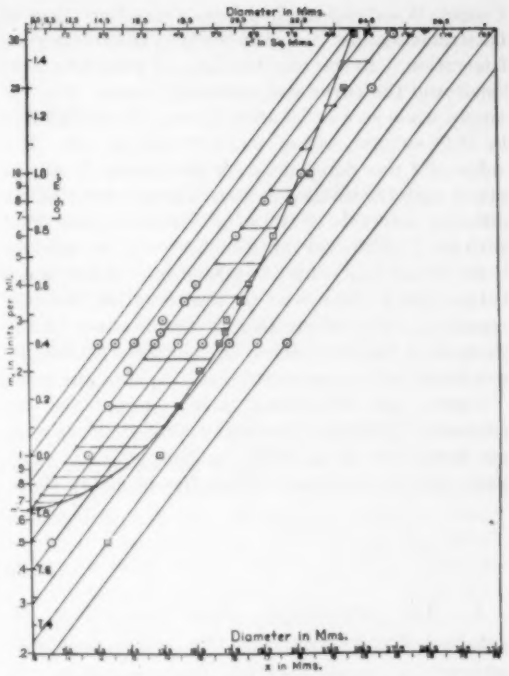

Fig. 3.—Cylinder plate assay. Cooper–Woodman standard curve. Log m_0 *versus* x^2. Circular symbols represent values of x^2. Values of log m_0 *versus* x (square symbols).

In Fig. 3, based also on Table I, the concentration of bacitracin is plotted on a logarithmic scale against values of x^2 on a linear scale. The resulting graph is shown by a straight line established by points which are designated by the circular symbols. On this curve fairly good correlation exists over a range of inhibition zones from 14.5 to 21.0 mm., which correspond to approximately 1.5 to 8 units per ml. Standard curves for other values of the 2.5 units per ml. standard have been indicated on this graph by lines parallel to the "17-mm." curve.

In order to calculate values directly from this graph it is only necessary to plot corresponding values of zone diameters from the "17-mm." curve on a suitable overlapping linear scale as shown in Fig. 3 by the curved line. Then from values of the zones of inhibition read on the lower scale one may read units directly on the logarithmic scale. It is of interest to note here that if values of x are plotted on the linear scale against log m_0, as has also been done in Fig. 3, a straight line relationship is again found to exist. In this instance the equation may be expressed as $\log m_0 = b'x + \log a'$.

Although the Cooper–Woodman formula is of theoretical rather than practical interest it has been used in our laboratory to calculate a chart for the assay of bacitracin. The work involved was cumbersome, however, and the method has no advantage over the simpler F. D. A. procedure.

The Coefficient of Diffusion.—The coefficient of diffusion of bacitracin was determined by the

Cooper–Woodman method from a standard curve of bacitracin against *S. aureus* without preliminary refrigeration. In this case the zones of inhibition were small and the curve was extremely steep. For example, when m_0 was 5.0 units per ml., x^2 was found to be 18.06 sq. mm. and m' was 0.90 unit per ml. The value of t was determined by incubating *S. aureus* plates with the unfilled cylinders in position. After different intervals of time the cylinders were filled with the 2.5 units per ml. standard. In this manner it was found that with an elapsed time of five hours before filling there was no inhibition of the test organism. By substitution of these values in the theoretical equation, the coefficient of diffusion, D, was found to be approximately 0.53 sq. mm per hour.

Cooper and Woodman have reported the coefficients of diffusion of crystal violet as 0.30 sq. mm. per hour and of penicillin as 1.06 to 1.57. The value 0.53 for bacitracin is therefore probably about one-half to one-third of that of penicillin. This difference in diffusion rates is in line with the pharmacological findings that bacitracin is not absorbed into the blood stream from the tissues as rapidly nor excreted as fast as penicillin. For example, significant concentrations of bacitracin persist in the blood stream as long as seven to eight hours after a single subcutaneous or intramuscular injection, while penicillin blood levels drop in two or three hours. Scudi (7) reports that urine levels of bacitracin may persist for at least 24 hours after a single injection while penicillin disappears in six to eight hours. Thus the cup plate method may permit some generalizations to be made on the movement of the antibiotic through the tissue cells. In this respect it is unwise for investigators searching for new antibiotics to discard all organisms which produce only a small zone of inhibition on agar plates.

SUMMARY

1. The antibiotic, bacitracin, can be assayed by essentially the same methods which are in general use for penicillin. A turbidimetric and a cylinder plate technique are described.

2. Methods for establishing standard curves and charts for the cylinder plate assay are described and the nature of the formula of the graph is discussed in detail.

3. The coefficient of diffusion calculated from the Cooper–Woodman formula was found to be about one-half to one-third of that of penicillin. This is in agreement with published pharmacological findings which show slower excretion rates for bacitracin than for penicillin.

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DISPENSATORY ERRATA ANNOUNCED

The attention of owners and users of the 24th Edition of the "United States Dispensatory" is directed to certain corrections of typographical errors in the first impression of the book.

Page 496—Line 15, column 2: The number 16 in this line should be changed to 6.

Page 794—The amount of antimony and potassium tartrate specified in the formula for Compound Opium and Glycerrhiza Mixture should be changed to read 0.24 Gm. instead of 24 Gm.

Page 1573—Line 2, column 2: Change "lucite" to "leucite."

—J. B. LIPPINCOTT COMPANY, *Publishers*

Ascaridol in Oil of Chenopodium*

By ALFRED HALPERN†

The methods proposed for the determination of ascaridol content of oil of chenopodium have been reviewed and the literature surveyed. The possibility of utilizing the oxidative property of ascaridol on ascorbic acid as an assay procedure has been investigated. There was no evidence of a stoichiometric relationship between the reactants. Emphasis is placed on the need for a re-investigation of the oxidative behavior of ascaridol because of the nonconformance among the various assays between the experimental and calculated values based on the theoretical explanation of these reactions.

OIL OF CHENOPODIUM has a relatively wide therapeutic use as a vermifuge. Smillie and Pessoa (1) have shown that ascaridol was the sole component of the oil which exhibits anthelmintic action against hookworm and round worm, for which the oil is generally used. A determination of the ascaridol content of the oil of chenopodium is therefore important from the therapeutic standpoint; also as oil of chenopodium commands a fairly high price, it is consequently further liable to adulteration. This was the case of the "synthetic" chenopodium oils to which attention was called by Schimmel and Company in their reports for 1919 (2).

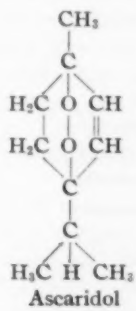
Ascaridol is unstable to heat and is liable to explode above 100°. It forms no known solid derivatives by which it can be characterized. It is capable of being reduced to the corresponding glycol by ferrous sulfate and to 1,4 terpin by hydrogen in the presence of palladium (3).

The U. S. P. X recognized the oil of chenopodium, but apart from physical constants there was no determination for ascaridol content.

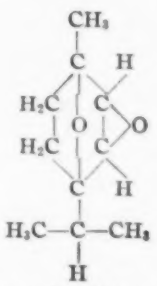
METHODS REVIEWED

Schimmel and Company (4) determined the ascaridol content of chenopodium oil by vacuum distillation to be 62–65% in the normal oil and 45–50% in the light oil. This method is slow and yields results obviously dependent upon the technique employed and which do not account for the ascaridol altered by the distillation process. Nelson (5) showed that ascaridol undergoes molecular rearrangement when heated to 150°. He further found that a bath temperature of 80° at 3–6 mm. pressure was sufficient for the terpene fraction. A bath temperature of 115° at 3–6 mm. pressure gave an even distillation of the ascaridol fraction. As a secondary check on the distillation method, Nelson employed a calculation of the ascaridol content of the oil by its density, assuming that the density of the terpenes was 0.8466 and that of ascaridol was 1.005. No other constituents were assumed present in the oil. The figures thus obtained run roughly parallel to those of the direct distillation. The limitations of this method are readily apparent and do not serve for an estimation of ascaridol content in an "unknown" oil.

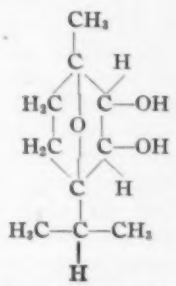
A rapid method of determination was suggested by Nelson (6) based on the solubility of the ascaridol portion in 60% acetic acid. The terpenes present in



Ascaridol



Ascaridol Glycol Anhydride



Ascaridol Glycol

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the oil of chenopodium were shown to be insoluble in 60% acetic acid. The method is simply to agitate 10 cc. of the oil of chenopodium with 60% acetic acid in a cassia flask. The neck of this flask is to be graduated in tenths. The flask is then filled to the mark with 60% acetic acid and allowed to settle or is centrifuged. The ascaridol content was determined from the difference in height between the original and treated oil columns.

This method of assay has many deficiencies: first, this method does not distinguish between ascaridol and the intramolecularly rearranged products (ascaridol glycol anhydride and ascaridol glycol) which are soluble in 60% acetic acid. Cineol, a component of oil of chenopodium, is also soluble in 60% acetic acid. Second, from the standpoint of technique, the time of shaking, the period of standing, the advisability of centrifuging, and the method of reading the oil column are left to the individual worker. Third, no method is given for determining the ascaridol content in oils heavier than the specific gravity of the 60% acetic acid. Such would be the case if carbon tetrachloride, another common vermifuge, were present.

Paget (7), after pointing out some of the above deficiencies of the method, suggested that the oxidizing property of ascaridol be used to determine the concentration. He devised a method based on the reduction of titanous chloride ($TiCl_3$), stabilized with hydrochloric acid. This method has an empirical factor taken from the average of several experiments, and was based on the mean value of 1 Gm. of ascaridol, reduced by 1.277 Gm. of titanous chloride. The author does not claim a high degree of accuracy for the method. He further states that the specific gravity was a useful guide to the ascaridol content, and that the solubility in 60% acetic acid was the most rapid and convenient for field work. For detection of highly adulterated samples the reduction technique or the distillation method was best.

In a later paper Nelson (8) recognized the limitations of the acetic acid solubility method, then official in the U. S. P. X. A sample containing 50% ascaridol, 20% turpentine, and 30% eucalyptol assayed as 85% ascaridol by the acetic method and as 43.2% by the Paget method.

A report of the committee (9) established to investigate the method of assay of oil of chenopodium for ascaridol content, by the Association of Official Agricultural Chemists, compared both methods of analysis (*viz.*, Nelson's and Paget's). The empirical factor of Paget was established as 1.284 (a difference of 0.007 from the original factor) and was arrived at as the mean of sixteen determinations on a highly purified ascaridol fraction. The difference between their factor and that of Paget was attributed to the different concentrations of hydrochloric acid used to stabilize the titanous chloride in both studies.

When the acetic acid method and the Paget method were used to determine the ascaridol content on known samples, two sets of results were obtained (9). In the first, the Nelson assay gave higher values than the Paget method. In the second, the reverse was noted. The high result of the Nelson method, in the first case, was accounted for by the presence of *p*-cymene, the principal constituent of the hydrocarbon fraction of the oil of chenopodium. Cymene and 60% acetic acid are partly miscible. Ascaridol is miscible with cymene in all proportions. Therefore, if an oil of chenopodium containing cymene was mixed with 60% acetic acid, the ascaridol was distributed over the two layers. Thus two conjugate ternary systems were produced, each containing ascaridol, cymene, and acetic acid. These two systems were in equilibrium with the other and the composition of each would depend on the concentration of the three components and the temperature of the mixture. In the Nelson assay, the cymene would always contain some ascaridol, the amount dependent upon the concentration of cymene in the oil. Thus, less ascaridol was accounted for by this method of assay. This was confirmed by a series of dilution studies on a highly purified ascaridol and cymene mixture.

Knaffl-Lenz and Hoffman (10) devised a method based on the color developed between ascaridol and hydrochloric acid. To a 1% solution of the oil in alcohol was added 1 cc. of concentrated hydrochloric acid. The mixture was allowed to stand a number of hours, six being considered sufficient. (However, better results were obtained on standing twenty-four hours.) The samples were then compared in a colorimeter with a standard solution of 100% ascaridol treated in the same way. Accurate readings were hard to obtain because of the presence of dark insoluble oil droplets in every mixture. (It was found necessary to dilute each sample in order to obtain a sufficient quantity for use in the colorimeter.) Furthermore, terpineol, an adulterant studied, was found to give an added color to the solutions. A solution of 50% ascaridol in terpineol gave a value of 55%.

Other color reactions that take place when ascaridol-containing oils are treated physically and chemically have been suggested as a measure of ascaridol (11, 12). These methods are more of a qualitative nature and do not lend themselves to an exact quantitative determination.

Cocking and Hymas (13) introduced a method of analysis based upon the liberation of iodine from potassium iodide by the organic peroxide ascaridol. The method required that 3 cc. of potassium iodide solution (83% *w/v*) be mixed with 5 cc. of concentrated hydrochloric acid and 10 cc. of glacial acetic acid and cooled to −3°.

To this solution, after cooling, was added 5 cc. of the oil sample prepared by diluting 2.5 Gm. of the oil to 50 cc. with 90% acetic acid. The flask was stoppered and allowed to stand in a cool place for five minutes. It may be left for ten minutes at temperatures below 10°. The contents were then titrated with 0.1 *N* thiosulfate solution. A blank was carried out precisely as described except that the final solution was diluted with 10 cc. of water before

titrating. It was found necessary to employ an empirical factor, the relation being 1 cc. of 0.1 *N* thiosulfate equals 0.00665 Gm. of ascaridol.

This method has several limitations. The authors claimed that three reactions occurred in sequence. Two moles of iodine were liberated from the acidified potassium iodide solution, followed by an unexplained liberation of iodine and then a reabsorption of iodine when the solutions were diluted. This method, on the basis of limited determinations, was found not to be well suited to high-grade oils. The chances of error were great unless rigorous specifications were followed. This method was adopted by the U. S. P. XII and N. F. VIII.

In comparing the iodine method with the Paget method, the former has the advantage of not requiring the precautions necessary to stabilize the titanous chloride and also does not require a neutral atmosphere. However, from the point of view of time it is the same as that of Paget's method and it also employs an empirical factor.

"Broughton felt that the iodine assay was worthy of further study, in spite of the number of factors that influenced the accuracy. Dafert and Capesius found it simple and accurate. Bodendorff felt that the development of an exact quantitative method for ascaridol based on an iodometric procedure is highly improbable" (14).

In an attempt to devise a biological method, Reindollar and Munch (15) came to the conclusion that no relationship exists between the lethal dose and ascaridol content as determined chemically. Knaffl-Lenz and Hoffman (10) reported similar results.

In view of the above difficulties for the determination of ascaridol, it was decided to investigate the behavior of ascorbic acid in the presence of ascaridol and oil of chenopodium. The organic peroxide presumably should oxidize the sensitive ascorbic acid, and since ascorbic acid could be fairly easily determined by titration with dichlorphenolindophenol the quantity of ascaridol present in a sample would be indicated by the amount of ascorbic acid oxidized.

EXPERIMENTAL

An ascaridol fraction was obtained by distillation of oil of chenopodium at 85–95° at 2–5 mm. Hg. This fraction was redistilled twice and corresponded to 59% of the quantity of oil taken. The specific gravity of this fraction at 20° was 0.998 and when assayed by the Nelson method corresponded to 100% ascaridol and 99.8% by the potassium iodide method.

A preliminary series of reactions with ascaridol and ascorbic acid were run to ascertain the oxidative effect of ascaridol. A rather wide variation of values was obtained. It was thought that the uneven distribution of the oil in the water was responsible for the variations observed. Alcohol was substituted for a large part of the water and the stability of the ascorbic acid in this medium was determined.

To 0.01 mole of ascorbic acid dissolved in 150 cc. of water, was added 450 cc. of 95% alcohol. The solution was kept in an ice bath at 0° throughout the study. At the indicated intervals an aliquot portion was removed and titrated with standardized dye.[1] The amount of ascorbic acid remaining was referred to the original dilution (Fig. 1). The stability of the system was thus established.

Determinations on the purified ascaridol fraction obtained by distillation were attempted.

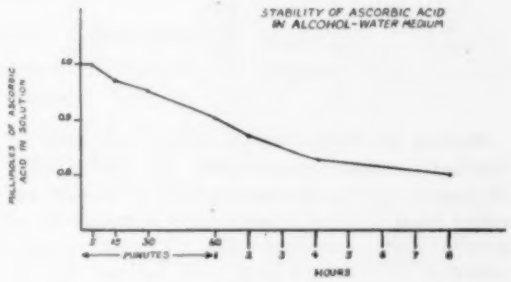

Figure 1

Method A.—One millimole of ascaridol in 15 cc. of 95% alcohol was mixed with a solution of 5 millimoles of ascorbic acid in 5 cc. of water. The mixture was cooled at 0°, in an ice bath, for ten minutes, with constant shaking. The mixture was diluted with 10 cc. of water and titrated with standardized dye. The amount of ascorbic acid oxidized by 1 millimole of ascaridol was thus determined (Table I). A blank was run without ascardiol.

TABLE I.—OXIDATION OF ASCORBIC ACID BY 1 mM OF ASCARIDOL[a]

Sample No.	mM of Ascorbic Acid Oxidized
1	1.6
2	1.4
3	1.8
4	1.6
5	2.0
6	1.4
	Av. 1.6 ± 4% A.D.

[a] Empirical factor = 1 mole of ascaridol oxides. 1.6 moles of ascorbic acid.

The determination was repeated at increased time intervals to determine whether the value obtained was influenced by the length of time the ascaridol was in contact with the ascorbic acid. The determination was carried out in the described manner except for the time interval (Table II). The length of time the ascorbic acid remained in contact with the ascaridol at 0° apparently did not influence its oxidation within certain limits. There was an appreciable increase in the oxidation of ascorbic acid at room temperature (24°). The time limit was arbitrarily maintained at ten minutes at 0°.

[1] Sodium Dichlorphenolindophenol, standardized by procedure given in the U. S. P. XIII, p. 620.

TABLE II.—INFLUENCE OF TIME ON THE OXIDATION OF ASCORBIC ACID BY ASCARIDOL

Sample No.	Time, Min.	Temp., °C.	mM of Ascorbic Acid Oxidized
1	5	0	1.4
2	10	0	1.8
3	15	0	2.0
4	20	0	1.7
5	25	0	1.4
6	30	0	2.1
7	(5 at 0) (plus 5 at 24)		2.3
8	5	24	2.6
9	10	24	2.8

Method B.—Following the above procedure for distilled ascaridol approximately 0.5 Gm. of oil of chenopodium was dissolved in 15 cc. of alcohol and mixed with 5 cc. of a solution of 5 millimoles of ascorbic acid in distilled water. The mixture was cooled in an ice bath to 0° and agitated for ten minutes. At the end of this time the mixture was diluted with 10 cc. of water and titrated with standardized dye solution (Table III).

TABLE III.—DETERMINATION OF ASCARIDOL BY ASCORBIC ACID METHOD

Sample No.	Sample Weight	mM of Ascorbic Acid Oxidized[a]	Equiv. mM of Ascaridol[b]	Ascaridol, Gm.	Ascaridol, %
1	0.4263	2.9	1.8	0.3027	71
2	0.3214	2.6	1.6	0.2764	86
3	0.4210	2.2	1.4	0.3283	78
4	0.2170	1.8	1.1	0.1802	83
5	0.4983	3.34	2.1	0.3587	72

[a] Corrected with blank determination run simultaneously with experimental.
[b] Based on relationship derived from Table I.

This oil of chenopodium was then assayed by the acetic acid method (Nelson) and was found to contain 68.5% of ascaridol. This value was checked by the iodine method and was found to contain 66.8% of ascaridol.

The abnormally high results obtained from the determinations made on the oil of chenopodium indicated that there was some additional factor present in the oil that oxidized the ascorbic acid. An attempt was made to determine the activity of the fractions obtained by distilling the oil under reduced pressure. The fraction distilling at temperatures up to 80° under 3–5 mm. pressure was separated. On assay by the iodine method, this fraction was found to contain 6% of ascaridol. This fraction was further purified by washing with 60% acetic acid and then neutralized by washing with 5% sodium bicarbonate solution. On assay of this sample no ascaridol was found by either the iodine method or the acetic acid method. This fraction was then assayed with ascorbic acid in the manner described above. Approximately 0.5 Gm. of this sample was used (Table IV).

DISCUSSION

The oxidation of ascorbic acid by ascaridol did not proceed in the expected mole-mole relationship. There was some extraneous factor present in this system that was also reacting with the ascorbic acid. Since we sought a means of determining the ascaridol content of oil of chenopodium that would be applicable to the usual laboratory equipment, specially prepared apparatus and reagents were not used. Rather, the routine glassware found in any well-equipped laboratory was cleaned in the usual manner, rinsed with distilled water, and air-dried before use. The distilled water was delivered from a block tin system and met the U. S. P. XII requirements. It is therefore necessary to point out the sensitivity of ascorbic acid to trace concentrations of metals. While it was felt that the influence of such a variable was kept at a minimum, as evidenced by the stability study of the system and in the blank determinations run simultaneously with the assay, this factor must be considered in a valid discussion of ascorbic acid oxidation.

The nonconcurrence among the values obtained as a result of the determinations made on the distilled ascaridol indicated a behavior of ascaridol that was not quite understood. It is of interest to note that none of the oxidation-reduction reactions thus far investigated for assay purposes afforded results that agreed stoichiometrically. The consistent failure among the various assays to obtain values that correspond to the theoretical, based on a mole-mole relationship, necessitates a re-examination of the mechanism and resulting products from these reactions. The reproducibility of the empirical values obtained from the iodine and titanous chloride assays indicates that these reactions behave in a systematic manner and that a factor, as yet unexplained, was the determinant in these reactions.

The evidence of some oxidation of ascorbic acid by the terpene fraction of oil of chenopodium was rather interesting. It was impossible to state on the basis of these limited results whether the latent oxidation was due to one of the constituents of this fraction (i.e., *p*-cymene) or to some rearranged product of ascaridol which resulted from the distillation procedure. However, the values obtained from these experiments (Table IV) do not account

TABLE IV.—OXIDATION OF ASCORBIC ACID BY TERPENE FRACTION

Sample No.	Sample Weight	mM of Ascorbic Acid Oxidized[a]	Equiv. mM Ascaridol[b]	Ascaridol, Gm.	Ascaridol, %
1	0.4215	0.32	0.20	0.0337	8
2	0.5126	0.50	0.31	0.0517	10
3	0.4613	0.80	0.50	0.0846	12
4	0.4461	0.21	0.13	0.0223	5
5	0.3314	0.23	0.14	0.0232	7

[a] Corrected with blank determination run simultaneously with experimental.
[b] Based on relationship derived from Table I.

for the difference in ascaridol content as determined by this method and the others on the same oil.

The extreme variation among the values obtained on the distilled ascaridol and also among the different samples of the same oil of chenopodium invalidated further investigation with other preparations from different sources.

The reagents used to determine the active ingredient of a drug should evidence a measure of specificity for that agent. The results of this investigation indicate no such specificity. The application of the above method to the determination of ascaridol in oil of chenopodium must be viewed negatively for the above reasons.

SUMMARY

1. The methods proposed for the determination of ascaridol content of oil of chenopodium have been reviewed and the literature surveyed.

2. The possibility of utilizing the oxidative property of ascaridol on ascorbic acid as an assay procedure has been investigated.

3. There was no evidence of a stoichiometric relationship between the reactants. The wide variation of values that was obtained with distilled ascaridol and oil of chenopodium invalidates this method as an analytical procedure.

4. Emphasis is placed on the need for a re-investigation of the oxidative behavior of ascaridol because of the nonconformance among the various assays between the experimental and calculated values based on the theoretical explanation of these reactions. All described methods for the determination of ascaridol content employ an empirical factor. The results of the various assays are reproducible indicating a systematic constant order of reaction that is not explained by the theoretical considerations proposed for the reaction.

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WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation, 33rd, Federal and Dearborn Streets, Chicago, Ill., seeks information on sources of supply for the following chemicals:

Montanic acid
Glucoascorbic acid
Acenaphthylene
Cuscohygrine
Hemipinic acid
Hordenine
4-Hydroxyquinoline-3-carboxylic acid
4-Phenyl-2-aminobutyric acid
Undecyl aldehyde
Scopoletin
N-Methylnipecotic acid
Ethyl vanillate
Thiocytosine
Phosphatase, purified
Hydroxylamine-O-acetic acid hydrochloride
Methoxinine
d-Erythrose
d-Threose
17-Hydroxycorticosterone
11-Dehydro-17-hydroxycorticosterone
Stigmasterol
Maltobionic acid

A Quantitative Microscopic Study of Certain Commercial and Vegetable Drug Starches*

By ROBERTS L. PROPER† and H. W. YOUNGKEN, JR.‡

The existing methods employed for starch counting in starch mixtures have been studied and an improved method based upon the use of a saturated solution of zinc sulfate and a Levy blood counting slide is proposed. The selection of a suspending agent of close proximity to the specific density of starch, the latter being at the same time not altered, provided for easier handling of the sample, rapid counting, and more consistent results. Methods are described for extracting pure starch from the vegetable drugs belladonna, aconite, glycyrrhiza, and orris roots. Limitations and disadvantages of the proposed method are described.

THE occurrence of starch granules in cereal products and vegetable drugs is of prime importance to the microscopist who endeavors to identify such substances in certain mixtures. The microscopic identification of starch granules has as its basis a knowledge of the structural characteristics of each type of starch. Along with these morphological characteristics, the microscopist frequently has need to determine the amount of starch present in a given mixture by means of quantitative methods. The most acceptable methods for the quantitative determination of starch are based upon the enzymatic and chemical hydrolysis of the starch substance with subsequent formation of sugars and reduction of a copper-containing solution.

Quantitative methods for the determination of starch, other than the chemical methods, are recorded in the literature: for example those of Meyer (1, 2), Hartwich and Wichmann (3), Hart (4), Allen (5), Chamot (6), and Wallis (7). Schneider (8) and, more recently, Mendelsohn (9) have applied counting methods with some degree of success to the analysis of starch in baking powder mixtures and the microanalysis of vegetable drugs. In general, these methods involve the weighing of the starch mixture with subsequent suspension of the starch granules in a suspending medium and a microscopic counting of the starch by means of ruled slides. Such methods have generally fallen into disuse, due largely to certain technical difficulties such as occur during the handling of samples before counts are made in prescribed counting chambers and failures to establish a satisfactory comparison with the established chemical methods.

The problem forming the subject for this investigation arose following a desire to investigate the existing nonchemical quantitative methods of starch analysis and, where applicable, to devise a method which would give results more comparable with those from the accepted chemical methods.

MATERIALS AND METHODS

For the first phase of this study, the following commercial starches were used: corn, potato, wheat, and rice. Each starch was examined microscopically for the presence of altered granules, dextrins, fibers, and foreign substances.

Purified Talc and Purified Siliceous Earth were used in making unknown starch mixtures.

Starches were extracted from authentic fresh samples of four different vegetable drugs. These starches were used for the determination of reference standard starch counts for each drug studied. Such material was prepared from fresh root portions of Belladonna (*Atropa belladonna* L.), Glycyrrhiza (*Glycyrrhiza glabra* L.), Iris (*Iris pallida* Lamarck), and Aconite (*Aconitum napellus* L.).

The preparation from the fresh root portions of each plant was performed in the following manner.

The fresh materials were thoroughly washed and then peeled. The peeled drug was soaked in warm water (50°), containing 0.05% sulfurous acid (approx. 6%) until the drug material was thoroughly saturated. The sulfurous acid acted as a bleaching agent and removed most of any remaining pigments or tannins present in adhering rind. The material was again washed and then placed in a Waring

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† Abstract of a thesis presented by R. L. Proper to the Graduate School, University of Washington, in partial fulfillment of the requirements for the degree of Master of Science in Pharmacy.
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Blendor and thoroughly disintegrated into a pulpy mass. Enough water was added to facilitate removal of the pulpy mass from the Blendor. The milky mixture was then passed through a #60, #100, and #200 sieve in that order. The residue present on each sieve was washed until all starch had been removed. The milky starch-bearing liquid from the #200 sieve was placed in a large aspirator bottle and allowed to flow at the rate of approximately 30 drops per minute on a metal settling and washing trough. The trough was 5 feet long and fitted with baffle boards running transversely every 6 inches of its length. After all the starch-bearing water from the aspirator had passed down the trough, the residue (starch) remaining on the trough was washed slowly with pure water. This was done until, upon microscopic examination, only pure starch remained in the trough. The starch residue was removed from the trough with a small amount of water and strained through four thicknesses of fine grade silk bolting cloth. The residue was washed with water and the strained liquids were filtered by means of suction through a smooth hard filter paper (Whatman's No. 50). The starch was then dried by means of a small amount of 95% alcohol and then a small amount of ether. Finally the starch was removed from the filter and air dried in an evaporating dish.

Authentic samples of powdered vegetable drugs were used as unknowns in determinations involving vegetable drug starches. These samples were examined microscopically for adulteration and for alteration of starch granules. A hemocytometer slide was used as the counting chamber for the microquantitative procedures. This slide consisted of a Levy counting chamber with the improved Neubauer ruling.

The methods used for the determination of starch quantitatively by means of chemical analysis were those outlined in "Methods of Analysis of the Association of Official Agricultural Chemists" (10). The procedure was followed as described except that in place of malt diastase a saturated solution of Takadiastase was used.

EXPERIMENTAL

Part I. The Examination of Commercial Starches

Several samples of the common commercial starches of corn, wheat, potato and rice were examined to verify their authenticity. They were then mixed with common suspending agents, such as aqueous solution of acacia or mucilage of tragacanth. These were used in concentrations of 5%, 15%, 25%, and 50% solutions. Glycerin and water mixtures were used in concentrations of 10%, 25%, 50%, and 100% of glycerin. A chemically pure grade of triethanolamine was also used as a suspending medium. These agents were selected because of the use of each in the different starch counting methods as reported in the literature (2, 3, 6, 8, 9). The advantages and disadvantages of each medium were studied and found to be as follows: The vegetable gums in concentrations of 25% and 50% appeared to give the best suspension after considerable trituration and shaking. However, upon examination, the starch granules were observed to have a tendency to aggregate into small clumps. In the lower concentrations, the starch was not suspended satisfactorily for counting purposes. The glycerin and water mixtures and the triethanolamine were also found unsatisfactory for use in counting starch microscopically. The difficulty in obtaining a sufficiently homogeneous suspension for starch counting with the use of these suspending media was attributed in part to the fact that all suspending agents possessed too high a viscosity for satisfactory mixing with starches. On this assumption it was decided that a suspending medium having a viscosity lower than that of the above media and with a specific gravity as near that of starch as would be possible might produce a more homogeneous suspension. With an agent of this type, the suspension could then be made by placing the weighed sample directly into the medium, and shaking vigorously. The trituration necessary with the other suspending agents could thus be eliminated. In addition, the use of a more efficient suspending medium of this type would eliminate the need for making a starch paste in one receptacle and pouring the resultant mixture into another. It was noted that errors in starch counting were caused by both of these procedures. In this case the efficiency of the suspension would depend more upon the medium's specific gravity than upon its viscosity. Of those substances which have a specific gravity comparable to that of starch, concentrated solutions of certain inorganic salts appeared to be the most acceptable.

Among the inorganic salts used were potassium iodide, copper sulfate, copper nitrate, potassium carbonate, lead acetate, zinc chloride, and zinc sulfate. Of these salts a saturated solution of zinc sulfate was found to be the most suitable. The other salts either caused a rupturing and alteration of the starch granules or had a specific gravity that was too low for producing an efficient suspension of the starch.

Starch granules even after standing overnight in saturated solution of zinc sulfate showed no swelling or alteration effects of any kind. This agent was found to have a specific gravity almost exactly that of starch, *viz.*, approx. 1.5. A necessary precaution when using this solution was that to insure thorough suspension there must be vigorous shaking of the starch-zinc sulfate mixture.

A Levy blood counting slide was employed for making counts because of its standardization simplicity and ease in handling. The large corner squares were ideal for counting the larger starch granules, such as potato starch; the smaller central squares were used for the smaller starch granules, such as corn, wheat, and rice.

In order to ascertain the efficiency of a counting system it was necessary, first, to determine a stand-

ard count for a definite volume of a known dilution of the pure starch suspended in the zinc sulfate solution. This procedure was carried out in the following manner. A convenient amount of the pure starch, depending on the size and number of the granules present, was accurately weighed. These weights were from 20 mg. to 100 mg. The sample was then carefully transferred to a 10-cc. volumetric flask, and a few cubic centimeters of the saturated zinc sulfate solution added by means of a pipette. The flask was then shaken vigorously for two or three minutes and three drops of iodine test solution added. The flask was again shaken until the iodine was completely mixed and then zinc sulfate solution added to the 10-cc. mark. After thorough shaking a drop of the suspension was placed on the counting area of the Levy blood counting slide by means of a small capillary pipette. This pipette consisted of a piece of glass tubing drawn out into a tube of capillary dimensions. The first drop from the pipette was discarded and the second drop placed directly upon the counting area. The cover slip was then carefully laid in place taking care that no air bubbles were present and that none of the suspension had run over into the wells surrounding the counting area. If either of these errors occurred precautions were taken to clean the slide thoroughly, and to reload it with another drop of suspension from the capillary pipette.

In order to attain a standard figure for a given starch with any degree of accuracy it was deemed necessary to make at least eight separate counts. This necessitated loading and cleaning the slide and cover slip eight separate times. In this procedure there were certain precautions to be observed. These consisted of shaking the flask in order to resuspend any of the starch that had settled to the bottom and any suspension remaining in the pipette was blown back into the flask. Following this the pipette was wiped off on the outside and then inserted into the flask. The pipette was allowed to fill by capillary action and not by the usual method of applying suction.

After the eight counts were made the results were added and an average taken. These results were obtained when using the large squares by counting the starch granules in each square. The figures thus obtained were then added and divided by four to give the number of starch granules per 0.1 cu. mm. If the smaller central squares were used the total count of five was made and then multiplied by five thereby attaining the number of starch granules per 0.1 cu. mm. The figure obtained from either of these procedures was then accepted as being the standard for the specific starch and was used on all other determinations of that starch or mixtures containing it.

As an example the determination of the standard reference count for potato starch was performed in the following manner. The starch after having been suspended and obtained as previously described was placed on the hemocytometer and counted in the four large corner squares. The total number of starch granules in the four squares was then divided by four in order to obtain the number of granules in 0.1 cu. mm. This procedure was followed eight times and the average of the eight counts was found to be 147 granules per 0.1 cu. mm. This same procedure was followed in the case of starches with smaller granules except that five of the small central squares were counted, totaled and multiplied by five to obtain the standard count for the specific starch.

TABLE I.—STANDARD MICROQUANTITATIVE STARCH COUNTS FOR COMMERCIAL STARCHES

Name of Starch	Weight/10 Cc. of Suspension,[a] Mg.	Type Square of Counting Chamber Used	No. of Counts Made	Record of Individual Counts Made	Av./0.1 Cu. Mm.
Corn	100	Small	8	1900, 1725, 1800, 1725, 1950, 1925, 2000, 1775	1850
Wheat	50	Small	8	475, 460, 465, 485, 540, 530, 480, 520	494
Rice	50	Small	8	2390, 2310, 2175, 2185, 2250, 2240, 2195, 2375	2265
Potato	100	Large	8	150, 149, 144, 146, 127, 133, 147, 179	147

[a] Starch counts were compiled with the use of a Levy blood counting slide with the improved Neubauer ruling. Saturated solution of zinc sulfate was used as the suspending medium.

Table I shows the results of determinations made in an effort to obtain standard starch counts for pure samples of each of four commercial starches, *viz.*, corn, wheat, rice, and potato. The average obtained for each of these starches as shown in Table I was used as the standard count for that specific starch. In order to determine the efficiency of the above starch-counting method and to check the accuracy of the standard counts mixtures containing known percentages of these starches were made. The same procedure was carried out and the standard count was used in computing the per cent starch present in the mixture. For example if 100 mm. of a mixture of potato starch and Talc were weighed and suspended in zinc sulfate solution and

the average of eight counts was found to be 74 starch granules per 0.1 cu. mm., the mixture would contain 74/147 × 100 = 50.34% potato starch.

The mixtures used in these determinations were made with either purified talc or purified siliceous earth as the diluent. In the case of wheat starch a sample of whole wheat flour was used as the unknown. Figures 1 and 2 show the appearance of potato starch and corn starch in these mixtures as they appear on the counting slide under the microscope. In each case the weight of the sample used in making the count was the same weight as that used in determining the reference count for the specific starch in question.

In addition to making starch counts of these mixtures a chemical assay was performed on the same sample. This procedure served as a check on the relative accuracy of the count system. At the same time it was used as a basis for comparing the results obtained by use of both methods. The results of these determinations are shown in Table II.

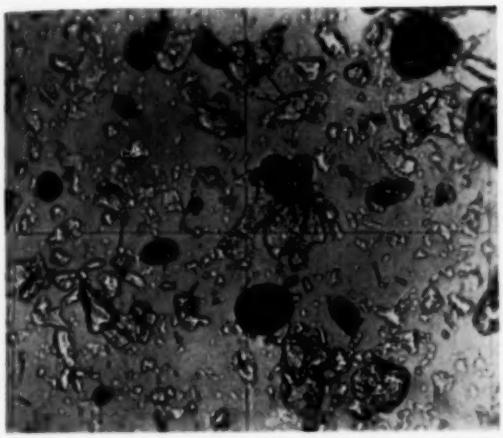

Fig. 1.—A mixture of Potato Starch in Purified Talc showing the starch granules to be counted in the large squares of the Levy blood counting chamber.

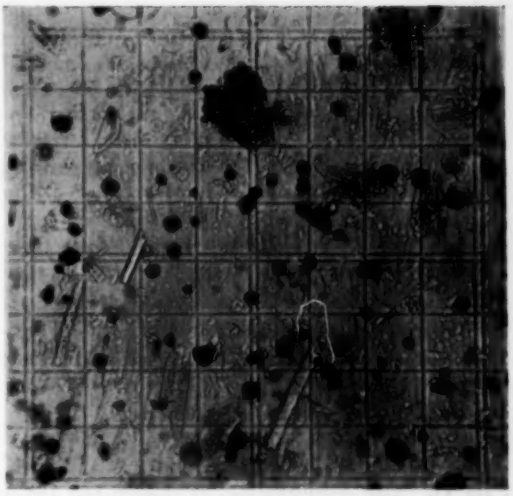

Fig. 2.—A mixture of Corn Starch in Purified Siliceous Earth showing the starch granules to be counted in the small squares of the Levy blood counting chamber.

These results show that in most cases the counting method and the chemical method compared favorably with one another and with the amount of starch that was actually present in the mixture.

Table III shows the results obtained in an attempt to determine the amount of potato starch in a known mixture of this starch with whole wheat flour. This determination indicated 0.54% more potato starch than the amount actually added to the whole wheat flour. However, the result is thought to be within the limits of experimental error expected from microscopic or chemical procedures. It was found difficult to differentiate completely between small potato starch granules and many wheat starch granules of the same size. This difficulty resulted in more time being required to complete the method than normally required with single starch mixtures. It was felt that a starch-counting pro-

TABLE II.—MICROQUANTITATIVE AND CHEMICAL STARCH ANALYSES OF COMMERCIAL STARCHES

Type of Starch	Diluent	Actual Starch, %	No. of Counts	Starch from Individual Counts, %	Av. by Counting, %	From Chemical Analysis, %	Av. of Chemical Analysis
Potato	Talc	10	8	10.03, 13.09, 8.33, 10.70, 9.40, 10.20, 9.40, 9.50	10.08	11.08 10.48 10.46 11.21	10.80
Corn	Siliceous Earth	25	8	27.83, 21.35, 26.75, 21.89, 24.59, 23.51, 27.59, 25.94	24.93	23.61 24.22 24.65 22.48	23.74
Wheat	Whole wheat flour		8	47.57, 48.58, 53.64, 52.63, 57.69, 51.61, 49.71, 53.47	51.86	52.72 49.16 51.27 51.05	51.03
Rice	Siliceous Earth	10	8	11.70, 11.20, 11.60, 10.90, 11.50, 11.00, 11.40, 11.50	11.30	10.92 8.95 10.26	9.61 8.95

cedure using mixtures of two or more starches would be applicable only in cases wherein starch granules of the same size could be accurately differentiated by means of definite morphological tests not investigated further.

TABLE III.—MICROQUANTITATIVE STARCH COUNTS —WHEAT FLOUR MIXTURE

Potato Starch Present in Whole Wheat Flour, %	Whole Wheat Flour Present, %	No. of Counts Made	Potato Starch from Individual Counts, %	Av. by Counting, %
10	90	8	7.4, 12.4, 9.1, 12.5, 9.7, 13.2, 9.52, 10.5	10.54

Part II. The Examination of Vegetable Drug Starches

The micro-quantitative starch counting method was applied to the examination of several powdered vegetable drugs in much the same manner as with the commercial starches. The drugs used in these determinations were Powdered Belladonna Root U. S. P., Glycyrrhiza U. S. P., Aconite N. F., and Orris Root N. F. Prior to counting the starch granules in these drugs it was necessary to obtain the pure vegetable drug starch in order to determine the standard starch count for each starch. These starches were obtained from fresh material by means of the washing process previously described. It was found that the fresh underground root portions of these plants were more satisfactory for use during this procedure than were the dried commercial drug materials.

Each starch from fresh drug sources was carefully examined for purity and alteration. Then the starches were weighed, suspended in the saturated solution of zinc sulfate, and counted in the same manner as were the commercial starches. The results obtained in determining the standard counts for these four vegetable drug starches are shown in Table IV. These results were used to calculate the

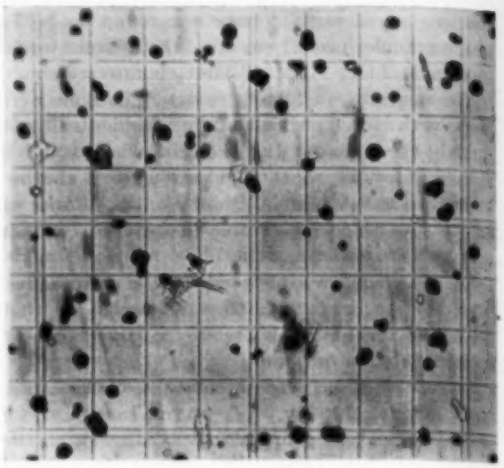

Fig. 3.—Powdered Belladonna Root showing the starch granules to be counted in the small squares of the Levy blood counting chamber.

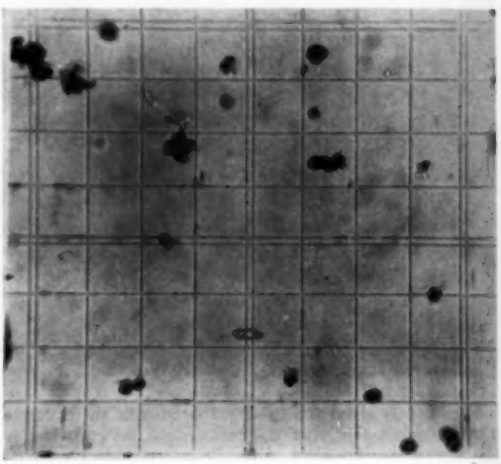

Fig. 4.—Powdered Orris Root showing the starch granules to be counted in the small squares of the Levy blood counting chamber.

TABLE IV.—STANDARD MICROQUANTITATIVE STARCH COUNTS FOR VEGETABLE DRUG POWDERS

Name of Starch	Weight/ 10 Cc. of Suspension,[a] Mg.	Squares of Levy Counting Slide Used	No. of Counts Made	Individual Counts	Av./0.1 Cu. Mm.
Belladonna	50	Small	8	1915, 1865, 2170, 2000, 2095, 2005, 1970, 2035	2007
Glycyrrhiza	20	Small	8	2365, 2495, 2415, 2510, 2100, 2165, 2580, 2430	2382
Aconite	25	Small	8	1220, 1160, 1340, 1400, 1165, 1180, 1165, 1220	1231
Iris (orris)	25	Small	8	470, 490, 520, 530, 655, 465, 650, 595	547

[a] A saturated solution of zinc sulfate was used as the suspending medium.

results of all subsequent determinations of each powdered drug containing that starch.

Following this, samples of the commercial powdered vegetable drugs, belladonna, glycyrrhiza, aconite, and orris roots, were counted and chemically assayed in order to determine the amount of starch present in each drug. The results of these determinations, both by the counting method and by the chemical method, are shown in Table V. Figures 3 and 4 show powdered belladonna root and powdered orris root as they appeared on the counting slide.

DISCUSSION

As a result of these determinations of commercial and vegetable drug starches, certain difficulties and precautions arise. Great care must be exercised when loading the counting chamber to insure that the starch granules are dispensed evenly. Occasionally some of the suspension on the outside of the pipette will drop on the slide causing an uneven dispersion of the starch granules. This can be remedied by wiping off the outside of the pipette with a clean towel before filling the counting chamber.

TABLE V.—MICROQUANTITATIVE AND CHEMICAL STARCH ANALYSES OF VEGETABLE DRUG STARCHES

Drug Used	Weight of Sample per 10 Cc. of Suspension,[a] Mg.	No. of Counts	Individual Counts, Starch, %	Av. Counting Starch, %	% from Chemical Analysis	Av. of Chemical Analysis, %
Belladonna	50	8	21.67, 30.17, 32.38, 26.90, 29.90, 27.90, 26.15, 27.40	27.80	28.75 27.32 28.75 28.40	28.30
Aconite	25	8	21.93, 23.55, 23.15, 24.77, 22.64, 21.43, 21.65, 24.16	22.91	20.09 17.92 18.22 19.91	19.04
Glycyrrhiza	20	8	16.75, 12.80, 14.69, 14.69, 15.95, 16.75, 16.16, 16.79	15.56	13.29 13.12 14.05 13.34	13.45
Iris (orris)	25	8	30.17, 31.09, 43.91, 53.83, 30.17, 48.48, 45.73, 46.65	38.75	39.52 40.05 37.63 39.08	39.07

[a] Starch counts were made with a Levy blood counting slide. A saturated solution of zinc sulfate was used as the suspending medium.

As a result of the counting procedure it was found that the eight individual counts for each drug were variable but that the average of all eight counts compared closely with similar averages obtained from the use of the chemical procedure. Glycyrrhiza and aconite gave results which were considered to be somewhat higher than those obtained by the chemical method. It appeared significant that during the starch-counting procedure, using rice starch, the latter starch likewise showed a slightly higher count than noted following the chemical analysis method. Glycyrrhiza, aconite, and rice starches are examples of starch granules of small size, mostly 5 to 10 microns in diameter. The slightly higher results obtained when counting these starches were thought to be due to the inclusion of other small similar-appearing particles in the count besides starch, probably in most cases tiny plant leucoplastids.

However, regardless of this fact the per cent starch obtained with most of these drugs when using the microscopic counting technique was found to be within the range of 1% to 2% of that obtained when using the chemical method. Thus the utility of the microscopic method is apparent.

The first drop from the pipette may then be discarded. A thorough and vigorous shaking of the suspension will aid in obtaining the desired results.

As the counting area of the Levy counting slide is 0.1 mm. in depth, starch granules will settle to different depths. Therefore, in order to make an accurate count it is necessary to use the fine adjustment of the microscope very diligently throughout each counting determination. This is of prime importance so that the starch granules in the different planes of focus can be properly counted. If this is not observed it becomes evident that inaccurate and low counts will result.

Such a microscopic counting method for the quantitative determination of starch was found to be both time- and labor-saving. This fact would be of great significance to the microscopist in the case of the above starch mixtures, if he desires to determine the amount of starch in a given mixture with a limited amount of time and effort and without resorting to the time-consuming and laborious chemical methods. However, it is felt that such a method will give most consistent results only when used for mixtures containing one type of starch and wherein most of the starch granules measure more than 10 microns in size.

SUMMARY

A study of several quantitative microscopic methods for analyzing commercial starches in mixtures and for the similar analysis of starch granules in certain vegetable drug powders has been made.

A saturated solution of zinc sulfate U. S. P. was found to be superior to agents previously used as starch-suspending agents. This suspending medium, when used with a Levy blood counting slide, gave sufficiently even starch granule dispersion to provide for more consistent microscopic starch counting than previously reported.

The above method was found best applicable to the analysis of the starches from potato tubers, grains of wheat, rice, and corn, and from the vegetable drugs belladonna, glycyrrhiza, aconite, and orris roots.

All counts and percentages ascertained were compared with results obtained from an accepted quantitative chemical analysis procedure on the same sample. Comparisons are shown in Tables II and V and were considered to be favorable.

The advantages and limitations of the procedure have been described.

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Book Reviews

Experiences with Folic Acid, by TOM D. SPIES. The Yearbook Publishers, Inc., Chicago, 1947. 110 pp. 15 x 23 cm. Price, $3.75.

That folic acid can control macrocytic anemia is now an established fact. Folic acid's place in nutrition and clinical science has changed so rapidly that a confusing literature is the result. Dr. Spies lists 80 references in this brief account.

Experiences with Folic Acid is an account of the clinical evaluation of folic acid largely by Dr. Spies and his staff. In addition to the information it contains, it could well serve as a guide to those interested in clinical research. Medical literature is so full of uncontrolled and poorly controlled clinical studies, that a scientific approach is indeed refreshing.

Since folic acid is so "new," obviously this account cannot be definitive, but it will serve as a helpful guide until research can crystallize more clearly.

Organic Analytical Reagents, by FRANK J. WELCHER. Vol. IV. D. Van Nostrand Company, New York, 1948. xiii + 624 pp. 14.5 x 23 cm. Price, $8; series price, $7.

Volume IV of *Organic Analytical Reagents* follows the same general pattern of previous volumes and shows the same meticulous care in its preparation. [For review of Volumes I and III see THIS JOURNAL, **37,** 44(1948).] The compounds covered in this volume are acidic nitro compounds, the arsonic acids, dithiocarbonates, xanthates, miscellaneous sulfur compounds, sulfonic and sulfinic acids, selenic acids, alkaloids, diazonium compounds, carbohydrates, miscellaneous natural substances, lake-forming dyestuffs, hydroxyanthraquinone and miscellaneous dyes, and dyes used in the detection of nitrites.

An index of names and synonyms of organic reagents and an index of the uses of organic reagents complete the volume.

A more helpful treatise on analytical chemistry than these four volumes has not appeared in years.

Pharmaceutical Preparations. By GEORGE E. CROSSEN and KARL J. GOLDNER. The Lea and Febiger Co., Philadelphia, 1948. 250 pp. 15.2 x 24 cm. Price $4.

The second edition of this text is a concise basic volume for students of pharmacy who have had preliminary training in chemistry and fundamental laboratory techniques and who are ready to consider official pharmaceutical preparations. It is a helpful supplementary outline of the official preparations which should be given in the course of galenical pharmacy in the colleges of pharmacy.

The authors have brought this edition into complete accord with the latest editions of the U. S. P. and the N. F. There are listed both old and new synonyms of the preparations, with added explanations for special steps which should be followed in the manufacture of some official preparations.

Each of the various classes of preparations is discussed separately in order to provide the best form of continuity. Valuable background material is furnished in the brief summaries of the Harrison Narcotic Act and the section of the Federal Food, Drug, and Cosmetic Act pertaining to "dangerous drugs" which is of interest to the pharmacist.